# Investigations on life-cycle and host specificity of the Malacosporea (Myxozoa)

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# II. List of Abbreviations

| | |
|---|---|
| **DAB** | 3,3-Diaminobenzidine tetrahydrochloride |
| **x *g*** | multiples of gravity |
| **H&E** | haematoxylin and eosin stain |
| **igG** | immune globulin gamma |
| **LAMP** | loop-mediated isothermal amplification |
| **MS222** | Tricaine-methane-sulphonate |
| **no.** | number |
| **PCR** | polymerase chain reaction |
| **PKD** | proliferative kidney disease |
| **PKX** | unknown proliferative kidney disease organism; old designation for *T. bryosalmonae*, before its systematic position had been clarified |
| **rDNA** | ribosomal deoxyribonucleic acid |
| **PBS** | phosphate buffered saline |
| **SE** | standard error of mean |
| **SEM** | scanning electron microscopy |
| **SPF** | specific pathogen free |
| **sp./spp.** | species |
| **S-T-doublet** | cell doublet of a secondary cell enclosing a tertiary cell |
| **TBS** | Tris buffered saline |
| **TEM** | transmission electron microscopy |
| **vs.** | versus, against |
| **wpe** | weeks post exposure |
| **v/v** | volume per volume |
| **w/v** | weight per volume |

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# 1. GENERAL INTRODUCTION

## 1.1 Phylum Myxozoa

According to present knowledge, the phylum Myxozoa Grassé, 1970 comprises about 2,000 species (Lom & Dyková 2006). Typical for this endoparasitic group is a life-cycle involving both invertebrate and vertebrate hosts (Markiw & Wolf 1983). The latter is usually a teleost fish, but infections with myxozoans have also been recorded in amphibians, reptiles (Eiras 2005), birds (Bartholomew *et al.* 2008) and mammals (Friedrich *et al.* 2000; Prunescu *et al.* 2007). Most common invertebrate hosts are tubificids (Annelida) for Myxosporea Bütschli, 1881, and freshwater bryozoans (Bryozoa; Phylactolaemata) are typical hosts for members of the class Malacosporea Canning, Curry, Feist, Longshaw and Okamura, 2000. Some of the fish-parasitic myxozoans can cause economically important diseases in marine and freshwater fish. Important examples are Whirling Disease of salmonids fish (*Myxobolus cerebralis*), Swimbladder Inflammation of carp (*Sphaerospora renicola*) the proliferative kidney disease (PKD) of salmonids (*Tetracapsuloides bryosalmonae*) in freshwater, and myoliquefaction in marine fish (*Kudoa* spp.) (El-Matbouli *et al.* 1992, Kent *et al.* 2001, Lom & Dyková 2006).
The most important characteristics of myxozoans are multiplication by endogeny, lack of centrioles during cell division, secondary cell formation, formation of spores in plasmodia or pseudoplasmodia, and their multicellular spores featuring polar capsules for attachment to the respective host (Kent *et al.* 2001, Canning & Okamura 2004). The spores consist of valve cells, capsulogenic cells and one to many sporoplasms. The sporoplasm contains one to many secondary cells, which are called sporoplasm cells. The sporoplasm penetrates the host, while the sporoplasm cells are the infective entities that start their development in the host. Sexual processes have been described in the invertebrate phase of myxozoan life-cycles, though there is a difference between the classes Myxosporea and Malacosporea. Meiotic divisions, formation of α and β gametes and their fusion could be observed for Myxosporea during sporogenesis in the annelid host. Capsulogenic, valvogenic and sporoplasmogenic cells develop from the resulting zygotes (El-Matbouli & Hoffmann 1998). All other stages in the life-cycle were found to be diploid (El-Matbouli *et al.* 1998). In the Malacosporea, the sporoplasms of the spores developing in the bryozoan host seem to remain haploid and it is not yet

clear whether and when fusion of two haploid cells actually occurs (Canning & Okamura 2004). Before the complex morphology of the spores and the differentiation of somatic cells were discovered, the Myxozoa were initially classified as protozoans, because of their reduced body plan. Nowadays their placement within the Metazoa is undoubted (Smothers *et al.* 1994). Spores are formed both in the vertebrate and the invertebrate host, but these two types of spores differ considerably in function and morphology (Canning & Okamura 2004). That is why the Myxozoa were initially subdivided into the Actinosporea Noble & Levine, 1980 and the Myxosporea, the former denominating the invertebrate phase and the latter the developmental stages in the vertebrate host. However, this classification is only of historical interest, because the discovery of the full life-cycle of *M. cerebralis* (Markiw & Wolf 1983, Wolf & Markiw 1984) showed that stages of Actinosporea and Myxosporea both represent only a part of the life-cycle of one myxozoan species (Kent *et al.* 1994). Meanwhile, complete life-cycles of more than 30 myxozoan species have been elucidated (Lom & Dyková 2006). Today, the Myxozoa comprise the two classes Myxosporea and Malacosporea (Canning *et al.* 2000, Lom & Dyková 2006). The largest part of the species-diversity of the Myxozoa is made up by the Myxosporea, while the Malacosporea comprise only three described species.

The systematic position of the Myxozoa is still controversial. Most results about the phylogenetic placement of this group are based upon the comparison of 18S ribosomal DNA (rDNA) sequences. Some researchers grouped the myxozoans within the Cnidaria (Siddall *et al.* 1995, Siddall & Whiting 1999), which is also supported by the strikingly similar morphology of cnidarian nematocysts and myxozoan polar capsules (Weill 1938, Siddall *et al.* 1995, Lom & Dyková 1997, Canning *et al.* 2008). Results of other studies indicate that myxozoans are basal bilaterians (Smothers *et al.* 1994, Katayama *et al.* 1995, Cavalier-Smith *et al.* 1996, Hanelt *et al.* 1996, Pawlowski *et al.* 1996, Schlegel *et al.* 1996, Winnipenninckx *et al.* 1998, Kim *et al.* 1999, Zrzavý & Hypša 2003). Additionally, ultrastructural studies on worm-shaped malacosporean stages in bryozoans revealed the presence of four longitudinal muscle blocks within the "worms", resulting in a body plan similar to some bilaterians (Canning *et al.* 2002). The latest molecular phylogenetic study including also sequences of protein coding genes showed that the Myxozoa most likely belong to the Cnidaria and are closely related to the Medusozoa. It could also be shown, that the bilaterian-like hox-genes used in a phylogenetic study of Anderson *et al.* (1998) were actually derived from the hosts and not from the myxozoan parasites (Jiménez-Guri *et al.* 2007a).

## 1.2 Class Malacosporea

The Malacosporea are differentiated from the Myxosporea by their soft spores without hardened valves, the presence of a special type of sporoplasmosomes, freshwater bryozoans as invertebrate hosts and the formation of spores in spherical or worm-shaped sacs in the body cavity of bryozoans (Canning & Okamura 2004). The first species of the class Malacosporea proven to belong to the Myxozoa was *Tetracapsuloides bryosalmonae*, the parasite causing PKD in salmonid fish. Prior to this finding, the developmental stages of this parasite in the fish host were referred to as PKX (for unknown proliferative kidney organism, Seagrave *et al.* 1980). Even though the similarity of the PKX-organism with myxozoan stages had already been noted several times by different authors (Kent & Hedrick 1985a, Kent & Hedrick 1985b, Feist & Bucke 1987), the identity of this parasite was uncovered not before 1999 (Saulnier *et al.* 1999). In the same year the link between developmental stages in fish and in bryozoans was discovered (Anderson *et al.* 1999a+b, Canning *et al.* 1999, Kent *et al.* 2000). Hence, it was known that *T. bryosalmonae* completes a part of its life-cycle in the coelomic cavities of freshwater bryozoans. The laboratory infection of fish by infected bryozoans could be achieved for the first time in 2001 (Feist *et al.* 2001) and the complete life-cycle of *T. bryosalmonae* was elucidated 5 years later (Morris & Adams 2006a). A second species, *Buddenbrockia plumatellae,* could be assigned to the Malacosporea in 2002 (Monteiro *et al.* 2002) and just recently *B. allmani*, the third malacosporean up to now was described (Canning *et al.* 2007).

The class Malacosporea was erected because of the fundamental differences of the myxozoans parasitizing freshwater bryozoans compared to all other members of this phylum. In this class, one single family, Saccosporidae, was created, originally comprising only the genus *Tetracapsula* (Canning *et al.* 2000). Later, the bryozoan parasite *B. plumatellae*, originally described by Schröder (1910), was found to be identical to *Tetracapsula bryozoides* described by Canning *et al.* (1996). This led to the omission of *T. bryozoides* as junior synonym of *B. plumatellae* (Monteiro *et al.* 2002, Canning *et al.* 2002). Because of morphological similarities to *T. bryozoides*, the parasite causing PKD initially was placed within the genus *Tetracapsula* (Canning *et al.* 1999). Later, in the course of the changes of nomenclature by Canning *et al.* (2002) it was renamed to *Tetracapsuloides*. Canning *et al.* (2008) again suggested to altering the name of the family Saccosporidae to Buddenbrockiidae, because the

old family name would be invalid according to the Code of Zoological Nomenclature as it is not formed from the stem of a generic name of this group.

### 1.2.1 Development and pathogenesis

The development and sporogony of the Malacosporea is to a great extent different from all other Myxosporea, especially regarding the formation of spores within sac-like structures in bryozoans. In the following four paragraphs the development in bryozoans and fish for both genera *Tetracapsuloides* and *Buddenbrockia* will be summarized in brief. Also, the effect of these parasites on the respective hosts will be addressed.

#### *1.2.1.1 Genus Tetracapsuloides: Development in the bryozoan host*

Freshwater bryozoans comprise about 41 to 60 known species (Wood 1989, Wood 2000) worldwide. They can be found attached to submerged surfaces in lakes and streams. Bryozoans are sessile animals that form colonies by budding and branching. The individuals of a colony are interconnected by a common coelomic cavity. They capture small particles, algae and other microorganisms by ciliated tentacles creating a water current towards the mouth opening (Wiebach 1960). The coelomic fluid in the body cavity of bryozoans is circulated by cilia on the inside of the peritoneum (Wood 1991). Besides sexual reproduction, which rarely occurs in some species, freshwater bryozoans are able to reproduce asexually (Karlson 1992). To this end, durable stages, so-called statoblasts, are formed. Statoblasts consist of germinative tissue enclosed within a hard shell and can bear unfavourable conditions after death of the colony (Mukai 1982). Malacosporeans are important parasites of bryozoans and infections with these parasites could be found in a wide variety of bryozoan species (Canning & Okamura 2004).

The process of infection of bryozoans by fishmalacospores of *Tetracapsuloides bryosalmonae* is not described yet. The earliest known phases of infection are amoeboid stages attached to peritoneum, retractor muscle and all other surfaces inside the bryozoans. At this stage of development, frequent cell divisions were observed (Morris & Adams 2006b). It was reported that the infection can be maintained in this state for up to 6 weeks (Tops & Okamura 2003). The further course of development from single cells to sporogenesis in spore sacs was described in great detail macroscopically by McGurk *et al.* (2006a) and microscopically by Canning *et al.* (2000) and Morris & Adams (2007a). Presporogonic cells aggregate and

circulate in the coelomic fluid of the bryozoans, which can be seen in live colonies under a dissecting microscope. These cells differentiate to mural cells forming gap junctions and adherens junctions to neighbouring cells making up the sac wall, and presporogonic cells inside of this spherical structure. In contrast to this interpretation, Canning *et al.* (2000) hold the view that cell divisions rather than aggregation of single cells are responsible for the growth of the sacs. The presporogonic cells further differentiate to sporogonic and stellate cells. Meiotic divisions were noted in those cells that later become the sporoplasm of the spore (Canning *et al.* 2000). Mature spores form by aggregation of stellate and sporogonic cells. They consist of two haploid sporoplasms including one secondary sporoplasm cell each (Canning *et al.* 2000), four capsulogenic cells and eight valve cells (McGurk *et al.* 2005b). Spore sacs grow up to a size of 200-350 µm, rupture and release the spores into the coelomic fluid of the bryozoans. Within 24 h, the spores are released from the bryozoans to the surrounding water by an unknown way, possibly the vestibular pore (Morris *et al.* 2002a). The whole development, with the first observable unicellular stages up to spore release, takes about 5 days and is temperature dependent (Tops *et al.* 2006). Obviously, bryozoans can pass several successive sporogonic cycles, although it was observed that infected parts of the colony are separated from the remaining colony and die (McGurk *et al.* 2005a). In electron microscopy, direct damage of the bryozoan retractor muscle caused by the development of Malacosporea was observed (Morris & Adams 2007a). Additionally, the overt parasite development inhibits statoblast production and colony growth (Morris & Adams 2006a, Tops *et al.* 2009). Parasite transmission to alternative habitats can also occur by fragmentation and reattachment of bryozoan colonies, which is common for *Fredericella sultana*-colonies (Morris & Adams 2006c).

#### *1.2.1.2 Genus Tetracapsuloides: Development in the fish host*

*Tetracapsuloides bryosalmonae* can infect a wide variety of salmonid fish. Most affected are species of the genera *Salmo* and *Oncorhynchus*, but also *Salvelinus* species (Hedrick *et al.* 1993, El-Matbouli & Hoffmann 1994). Severe outbreaks of the disease were also noted in grayling (*Thymallus thymallus*) (Hoffmann & Dangschat 1981). Northern Pike (*Esox lucius*) is the only non-salmonid fish species, in which extrasporogonic stages similar to those of *T. bryosalmonae* were found, although numerous non salmonid species from PKD-endemic waters were screened (Seagrave *et al.* 1981, Morris *et al.* 2000a).

The spores released from parasitized Bryozoa most likely enter the fish through the gills (Morris *et al.* 2000b, Holzer *et al.* 2006) or the skin (Longshaw *et al.* 2002), while the blood stream was considered to be the most probable route to the target organs (Kent & Hedrick 1986, Morris *et al.* 2000b, Holzer *et al.* 2006). The parasite develops mainly in the kidney but was also found in liver, spleen and other tissues like muscle, pancreas, gills and intestine (Ferguson & Adair 1977, Ferguson & Needham 1978, Kent & Hedrick 1985a). Multicellular stages of *T. bryosalmonae* were also detected in the blood vessels and the heart (Holzer *et al.* 2006). Transport of parasite stages to the kidney seems to occur mostly through the caudal vein, the portal system of the kidney and the peritubular capillaries and not via the efferent glomerular arterioles, as parasite stages were rarely found in the glomerular vessels of the kidney (Clifton-Hadley *et al.*1987). Proliferation of *T. bryosalmonae* in kidney, liver and spleen causes swelling of these organs by hyperplasia of the interstitial tissue and a granulomatous cellular reaction (Ferguson & Needham 1978, Clifton-Hadley *et al.* 1987). In severe cases, the disease causes anaemia due to replacement of blood stem cells by excessive multiplication of macrophages and lymphocytes in the kidney. Damage of blood-vessel endothelial tissue caused by parasite stages can lead to formation of thrombi and enclosure of kidney and liver vessels (Smith *et al.* 1984, Clifton-Hadley *et al.* 1987, Feist & Bucke 1993). Additional secondary infections and stress can cause high losses in aquaculture (Alderman & Feist 1985, Klontz *et al.* 1986, El-Matbouli & Hoffmann 1994). In heavily infected fish relatively unspecific symptoms like darkening of the body, swelling of the abdominal region, and uni- or bilateral exophthalmos can be observed (Ferguson & Needham 1978, Hedrick *et al.* 1984, Ellis *et al.* 1985, Clifton-Hadley *et al.* 1987).

The development of *T. bryosalmonae* in the kidney was described by Kent & Hedrick (1985a, 1986) and Morris & Adams (2008). In the interstitial tissue of the kidney, extrasporogonic parasite stages can be found as cell doublets (primary cell containing one secondary cell) measuring about 10 µm in diameter. These stages are typically enclosed within a host phagocyte. They proliferate by division of the secondary cell by open mitosis and subsequent division of the primary cell resulting in two cell doublets. By enclosure of one cell doublet by another a primary cell with two secondary cells and a tertiary cell inside of one of the secondary cells is formed (S-T-doublet). This S-T-doublet of cells migrates into the kidney tubules, and contact to the phagocyte is lost at this point. In the tubule-lumen sporogenesis proceeds in the primary cell. The latter is now called pseudoplasmodium, surrounding the spore-forming cells (sporoblast). In the end, the single secondary cell forms the valve cells

and the other secondary cell with the tertiary cell develop to capsulogenic cells and sporoplasms of the spore. The mature spores are about 12 × 7 µm in size and bear 2 polar capsules of about 2 µm length with 4 to 6 turns of their polar filament (Kent *et al.* 2000, Hedrick *et al.* 2004). The whole development beginning with the penetration of the fish to the presence of mature spores in the kidney tubules takes about 9 weeks in brown trout (Morris & Adams 2006a). Apparently, only few fishmalacospores are released by *T. bryosalmonae*-infected fish at one time, because only small numbers of spores were found in urine samples from infected fish (Kent & Hedrick 1986, Hedrick *et al.* 2004).

It was observed that fish become resistant against reinfection with *T. bryosalmonae* after surviving the disease (Ferguson 1981; Foott & Hedrick 1987). However, in some fish species sporogonic stages seem to persist after clinical infection and possibly continue to form spores chronically (Kent *et al.* 1998, Kent *et al.* 2000).

#### *1.2.1.3 Genus Buddenbrockia: Development in the bryozoan host*

While *Buddenbrockia allmani* develops in sac-like stages in bryozoans, similar to *Tetracapsuloides bryosalmonae*, the stages of *B. plumatellae* in the invertebrate host show a strikingly different body plan. Instead of spherical stages, this malacosporean species forms worm-shaped "sacs" with four blocks of longitudinal muscles. The bending and coiling movement of this parasite in the body cavity of the bryozoan host gives them a nematode-like appearance (Schröder 1912, Okamura *et al.* 2002). Various bryozoan species can be infected by *Buddenbrockia* spp. *B. plumatellae* can frequently be found in *Plumatella repens* (Schröder 1912, Morris *et al.* 2002a; McGurk *et al.* 2006b), but spore morphology of malacosporeans found in this bryozoan species provides evidence that it can be infected by different *Buddenbrockia* spp. Morris *et al.* (2002a) documented ornamented spores with a mean diameter of 19.0 µm in *P. repens* infected with worm-like malacosporeans. The spores observed by McGurk *et al.* (2006b), also released by a worm-shaped malacosporean in *P. repens*, were spherical and measured about 17.7 µm in diameter. The species *B. allmani* seems to be specific to the rare bryozoan *Lophopus crystallinus* (Tops *et al.* 2005; Canning *et al.* 2007). Worm-shaped malacosporeans were also found in colonies of *Fredericella sultana.* Sequence comparison of the 18S rDNA showed that this parasite might be a yet undescribed species (Tops *et al.* 2005). Spherical parasite stages were found repeatedly in the bryozoan *Cristatella mucedo* and their 18S rDNA-sequence was almost identical to the sequences of *B. plumatellae* (Canning *et al.* 1996, Okamura 1996, Monteiro *et al.* 2002). A recent study

provided strong evidence that sac and worm-shaped *B. plumatellae* are not morphotypes of the same species but represent two different species (Jiménez-Guri *et al*. 2007b). It has to be noted that in the worm-shaped malacosporeans, polar capsules were not only found in spores, but also in the cellular wall of the "worms" (Okamura *et al.* 2002).

The earliest malacosporean stages found in bryozoans were small uninucleate cells in the surface epithelium of the host (Canning *et al.* 2002). The sequential development of worm-shaped *B. plumatellae* in *P. repens* was described according to light microscopical observations by McGurk *et al.* (2006b). The first visible stages are small particles swirling in the coelomic fluid of the bryozoans. They attach to the peritoneum of the host and form "worms" within 10 days. These worms show bending and coiling movements in the bryozoan metacoel. In some cases, worms were able to leave degenerating colony parts and kept moving in water for up to 80 min (Canning *et al.* 2002), although this behaviour was not observed by McGurk *et al.* (2006b). After further 10 days mature spores could be detected in the body cavity of the bryozoans after rupture of the worms, which were later released to the surrounding water. Like for *T. bryosalmonae*, several consecutive periodical waves of parasite development could be observed in bryozoan colonies.

According to observations made by transmission electron microscopy (TEM), early unicellular stages of *B. plumatellae* show amoeboid movement similar to *T. bryosalmonae*, but are located inside of the peritoneum of the bryozoans. Necrotic changes of the host tissue surrounding the parasite stages were observed in infected colonies of *P. repens* (Morris & Adams 2007b). The latter observations could not be confirmed in *Hyalinella punctata* and *P. fungosa* infected with *B. plumatellae* (Canning *et al.* 2008). Aggregation and multiplication of single parasitic cells leads to development of the worm-like stage. Similar to *T. bryosalmonae*, an outer layer of mural cells is formed. Inside, cells differentiate to precursors of muscle blocks and sporogonic cells (Canning *et al.* 2002, Morris & Adams 2007b, Canning *et al.* 2008). The mode of spore formation of *B. plumatellae* in the invertebrate host is similar to that of *T. bryosalmonae*. The difference is that in *B. plumatellae* meiotic divisions seem to occur at the beginning of sporogony and therefore the whole resulting spore is haploid, not only the sporoplasms like in *T. bryosalmonae* (Canning *et al.* 2002, Canning & Okamura 2004, Canning *et al.* 2008). Morphologically, the spores of *B. plumatellae* and *T. bryosalmonae* developing in bryozoans are very similar.

Some detrimental effects of parasitism were observed in bryozoan colonies infected by *B. plumatellae*. Infected colonies separated infected areas by stenosis of the peritoneum

(Canning *et al.* 2002), though it seemed that spreading of the parasites could not be prevented (McGurk *et al.* 2006b). Also, statoblast production is diminished by infection with *B. plumatellae* (Morris *et al.* 2002a, Canning *et al.* 2002, McGurk *et al.* 2006b) and *B. allmani* (Hill & Okamura 2007). A long term ecological study on the bryozoan *C. mucedo* indicated that infection with *B. plumatellae* caused a decline of the bryozoan population at the site investigated (Vernon *et al.* 1996).

#### *1.2.1.4 Genus Buddenbrockia: Development in the fish host*

Hitherto, no fish stages are known for representatives of the genus *Buddenbrockia*. The only evidence for a possible infection of fish by malacosporeans other than *Tetracapsuloides bryosalmonae* was reported by Voronin (1993) and Voronin & Chernysheva (1993). These authors found developmental stages similar to those described previously from *T. bryosalmonae* in pillar cells of gills and blood vessel endothelia of brain and kidney of common carp (*Cyprinus carpio*). Although the origin of these stages was not clear, they suggest the existence of more fish-infecting malacosporeans. Hill & Okamura (2007) detected malacosporean DNA in statoblast-raised colonies of the bryozoan *Lophopus crystallinus*. These findings indicate that some malacosporean parasites might complete their life-cycles within the bryozoan host and do not require a fish as intermediate host.

### 1.2.2 Veterinary medical impact of Malacosporea

*Tetracapsuloides bryosalmonae* causes the proliferative kidney disease (PKD) of salmonid fish. This parasite can be found in Europe and North America and may cause high losses in trout aquaculture (Hedrick *et al.* 1993). In severe cases mortality of up to 100% has been recorded (Clifton-Hadley *et al.* 1984). According to evaluations by the British Trout Association made for the United Kingdom, losses ascribed to PKD were estimated to be as high as £2.5 million per year (Feist 2004). This economic impact of PKD makes this disease an important factor for aquaculture (Clifton-Hadley *et al.* 1986a). Additionally, PKD is suspected to be responsible for the decline of wild brown trout and salmon populations (Wahli *et al.* 2002, Sterud *et al.* 2007).

### 1.2.3 Treatment and control of PKD

No practicable treatment for PKD is available to date. The therapeutants used formerly were either shown to be toxic for fish or suspected to have negative effects on the health of the consumers (Alderman & Clifton-Hadley 1988; Wishkovsky *et al.* 1990; Morris *et al.* 2003). One possibility to reduce PKD-related losses is to delay the transfer of young fish from spring water tanks to the endemic water until autumn when water temperature is decreasing. As bryozoan populations decline under unfavourable conditions in autumn, only few spores of *Tetracapsuloides bryosalmonae* are present in the water at this time. Additionally, low water temperature prevents the clinical outbreak of the disease. In the subsequent year the fish usually show resistance against reinfection with this pathogen (Ferguson 1981, Foott & Hedrick 1987). Waterborne spores of *T. bryosalmonae* released by bryozoans are sensitive to mechanical damage and therefore are proposed not to withstand transport in the water over long distances. Removing the vegetation upstream the inlet of the fish farm, to reduce the number of bryozoan habitats and spore load in the water provides another possibility for prevention, but this measure will not be feasible in most cases (de Kinkelin *et al.* 2002).

## 1.3 Aims of the Present Study

The class Malacosporea is considered to be the most ancient group of the Myxozoa and probably represents the missing link between the entire phylum and its ancient metazoan ancestor. To date, detailed knowledge about life-cycles, range of susceptible fish species and the mechanisms of transmission is scarce for this class. A deeper understanding of malacosporean parasites would also help to clarify phylogeny, taxonomy and biology of the Myxozoa. Life-cycles of species of the genus *Buddenbrockia* are completely unknown, as past research was mainly focused on the infection of the invertebrate host and the development therein. Therefore, it was the main aim of the present study to gain more information on host parasite-interactions of malacosporean. Transmission experiments including attempts of a transfer to both hosts were conducted to identify fish species that are suitable hosts for malacosporeans. Furthermore, intra-specific differences in susceptibility of fish to malacosporean parasites were addressed. Additionally, the mechanisms of attachment and penetration of fish by malacosporean spores shed from bryozoans were investigated, as only

tiny details of this important aspect have been published so far. To enable experiments with these parasites under controlled conditions, setting up stable laboratory cultures of infected and non-infected bryozoans built the basis for this project. Thus, the results of this study provide yet unknown valuable facts about the biology of the Malacosporea. New possibilities for control and prevention of the economically highly important parasite *Tetracapsuloides bryosalmonae* are prospected to arise from these findings. The discovery of yet unknown malacosporean life-cycles will fundamentally increase the knowledge about the host-parasite interaction Malacosporea in general.

# 2. TRANSMISSION AND HOST SPECIFICITY OF *TETRACAPSULOIDES BRYOSALMONAE*

## 2.1 Introduction

For a long time, the complete life-cycle of *Tetracapsuloides bryosalmonae* was unknown. No mature spore stage could be found in the fish host and it was discussed, if fish might be accidental hosts instead of being obligatory for this parasite (Tops *et al.* 2004). Until now, no transmission within Bryozoa could be observed, as the spores released from bryozoans were found not to be infective for other colonies (Tops *et al.* 2004, McGurk *et al.* 2005a). However, there is some evidence that cryptic malacosporean stages might be incorporated into bryozoan statoblasts, being hereby transmitted to the next bryozoan generation (Hill & Okamura 2007). A direct transmission of *T. bryosalmonae* from fish to fish could be excluded (D'Silva *et al.* 1984). When a few years ago the transmission from infected brown trout (*Salmo trutta*) to bryozoans (*Fredericella sultana*) was achieved for the first time in a laboratory experiment (Morris & Adams 2006a), it was finally proven that fish are not accidental hosts for *T. bryosalmonae*. Bearing in mind that most salmonid species are susceptible to this parasite (Hedrick *et al.* 1993), the question arises, which species besides brown trout might be able to transmit the parasite to bryozoans. In their study, Tops *et al.* (2004) did not succeed in infecting bryozoans with *T. bryosalmonae*-infected brown trout, rainbow trout (*Oncorhynchus mykiss*) or chinook salmon (*O. tschawytscha*). Concerning brown trout, these results are contradictory to the findings of Morris & Adams (2006a). Therefore it is still uncertain whether other salmonids release infectious *T. bryosalmonae* spores and if so, under which conditions this occurs. Additionally, stages similar to those found in PKD-infected salmonids had been found in the kidney of Northern Pike (*Esox lucius*) (Seagrave *et al.* 1981, Bucke *et al.* 1991, Morris *et al.* 2000a), but the role of this fish species in the life-cycle of malacosporeans is totally unclear. In the trials of the present study, different fish species were tested for their capability to transmit *T. bryosalmonae* to bryozoans, thereby taking into account the possibility of vertical transmission of the parasite by cryptic stages in the statoblasts of the invertebrate host.

## 2.2 Materials and Methods

### 2.2.1 Fish

Rainbow trout were raised from eggs in the specific pathogen free (SPF) facilities of the laboratory. Brown trout, brook trout (*Salvelinus fontinalis*) and grayling (*Thymallus thymallus*) were obtained as SPF young of the year from the hatchery of the Landesamt für Umwelt, agency Wielenbach. Young of the year pike were obtained from a natural pond of the same hatchery. All fish were kept in aquaria with flow through of dechlorinated tap water. They were fed with commercial fish food, except for pike which were fed with life SPF rainbow trout. At the beginning of the experiment, the body lengths of the fish ranged from 9 to 15 cm.

### 2.2.2 Bryozoa sampling and culture

Colonies of bryozoans were collected at the river Lohr (50°01'08'' N; 09°32'43'' E). The sampling site is shown in figure 2.1A. Typically, the colonies were found in depths of 20 to 100 cm at roots of alder trees that reached into the water (Fig. 2.1B + C). According to colony and statoblast morphology, the bryozoans were determined as *Fredericella sultana* according to the key of Wood & Okamura (2005). For laboratory culturing, small pieces of roots with bryozoan colonies were glued to 12 cm plastic Petri-dishes with instant adhesive. Dishes were placed upside down in a rack (Fig. 2.1D) and placed in a 15 L bucket filled with dechlorinated, AquaSafe® (Tetra) treated tap water to remove possible heavy metal contaminations. Temperature varied between 15 to 18 °C. An airlift pump was used to keep the water moving slightly. Bryozoa were fed every second day with a mixture of five species of life algae (*Cryptomonas ovata*, *Cryptomonas* sp., *Chlamydomonas* sp., *Chlamydomonas reinhardii*, *Synechococcus* sp.). Pure cultures of each algae species were kept separately in 2 L flasks with aeration in autoclaved Guillard's WC-Medium (Guillard 1975) (Fig. 2.1E). Additionally, sterile stocks of algae were kept and propagated in 10 mL glass tubes to renew the flask cultures if necessary. For each feeding about 100 mL of each algae species was used per bucket. To foster bacterial growth as an additional food source, autoclaved mud was used as sediment in the bryozoan cultures. Water in the cultures was changed weekly.


**Figure 2.1:** Bryozoan sampling and culture. **A**. Bryozoan sampling site at the river Lohr. **B**. Roots of an alder tree, typical habitat for *F. sultana*. **C**. Enlargement of rectangle in B. Colonies of *F. sultana* can be seen on the roots (arrow). **D**. Rack holding Petri-dishes with bryozoan (*F. sultana*) laboratory cultures. **E**. Algae culture.

The bryozoans were checked daily with a dissecting microscope for visible infections with *Tetracapsuloides bryosalmonae*. The species *F. sultana* produces only statoblasts that are attached to the substratum and does not release floatoblasts to the water like most other bryozoan species. Therefore, it was necessary to dissect statoblasts from the colonies with fine

needles and forceps. The free statoblasts were then collected with a pipette and either stored at -20 °C if used for DNA extraction or kept for 1 or 2 weeks in tap water at +4 °C if used to raise new colonies afterwards. For this purpose, 10 to 30 statoblasts were placed in a Petri-dish with tap water and stored at room temperature for one week. After they had attached to the dish-surface, the Petri-dish was transferred to a 3 L aquarium and kept at 20 °C until the new colonies started to hatch. From this time on, feeding was started. When each colony had grown until it comprised at least 2 to 3 zooids they were placed in the normal culture tanks. Statoblasts were collected again from these laboratory-raised bryozoans and new colonies were raised to be used in the infection trials.

To ensure that the bryozoan colonies raised from statoblasts were parasite-free and no cryptic stages were present, a polymerase chain reaction (PCR) was conducted to compare statoblasts from these colonies with statoblasts from bryozoan colonies known to be infected with *T. bryosalmonae*. Four samples of 20 statoblasts each were washed with distilled water in a 1.5 mL reaction tube. To this end, the tubes were vortexed and subsequently centrifuged at 5,000 x *g*. The supernatant was discarded and the whole procedure was repeated 4 times. At the end, the water was removed and the pellet containing the cleaned statoblasts was frozen for DNA extraction.

### 2.2.3 Infection trials with salmonids

Rainbow trout, brown trout and brook trout were used for the infection trials. For infection with *Tetracapsuloides bryosalmonae*, four fish of each species were kept together continuously with infected colonies of *Fredericella sultana* for a period of 2 weeks in a 50 L aquarium. Water temperature was adjusted to 15 °C ± 1 °C with slight flow through of tap water. During this period, the bryozoan colonies were fed daily with about 100 mL of a dense culture of each algae species. After the cohabitation, the fish were separated by species in 20 L aquaria with flow through at a temperature of 15 °C ± 1 °C. To test if the exposed fish were capable of infecting bryozoans, two Petri-dishes, each with at least 3 statoblast-raised *F. sultana*-colonies com-prising about 10 zooids, were placed into each fish aquarium, at eight weeks post exposure (wpe). They were cohabitated with the fish for 8 h per day and were subsequently transferred to separate culture tanks containing an algae-suspension to allow feeding. As the cohabitation of bryozoans to exposed fish was continued for 6 months, it was not possible to keep the bryozoans continuously in the fish aquaria, because flow-through

water would have diluted the algae and would not have allowed survival of the bryozoans for such a long time. There were five additional dishes with statoblast-raised colonies kept without any contact to fish to serve as negative control. Both, the bryozoans exposed to fish and the control bryozoan colonies were checked every second day for a period of six months for developmental stages of *T. bryosalmonae*.

To check the infection status of the fish, one specimen of each group was anesthetised and dissected 3 wpe of the fish to infected bryozoans and a sample of the kidney was frozen at -20 °C for DNA extraction and PCR-analysis. An additional fish of each group was anesthetised and dissected 14 wpe to the bryozoans, and samples of the kidney were excised, fixed in formalin for light microscopy and frozen for DNA extraction. As the grayling were not available at the same time as the other fish, they were tested with the identical experimental setup 3 months later.

### 2.2.4 Infection trials with pike

As the pike used for the experiments were not SPF, kidney samples of four individuals of this population were taken for DNA extraction and testing by PCR for possible malacosporean infections. For the transmission experiment, four pike were exposed to infected bryozoans and sampled as described above.

### 2.2.5 DNA extraction and PCR

The statoblasts, fish and bryozoan tissues were homogenized in 1.5 mL reaction tubes with micropestles (Eppendorf) and lysed with proteinase K. Genomic DNA was extracted with a QIAamp® DNA Mini Kit (QIAGEN) according to manufacturers' instructions. An elution volume of 50 µL was used for 20 statoblasts, a part of a bryozoan colony comprising not more than 15 zooids and 25 mg of kidney tissue. As the extraction method used in the present study was not tested for bryozoan tissues or statoblasts, the success of the DNA extraction was tested by a Bryozoa-specific PCR. A new primer pair was developed (Tab. 2.1) according to 18S rDNA sequences of *Fredericella sultana* (GenBank™[1]; accession number: DQ221751; Wood & Lore 2004) amplifying a 730 bp long fragment of the bryozoan 18S rDNA.

[1]National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/Genbank/

**Table 2.1 Primer sequences**

| *Primer* | *Sequence* | *Reference* |
|---|---|---|
| bryo 18S-F | 5' - CTC TAG GCT GGC GGT CC - 3' | present study |
| bryo 18S-R | 5'- CGA CCG TCG GAG ATC GGT - 3' | |
| 5F | 5'- CCT ATT CAA TTG AGT AGG AGA - 3' | Kent *et al.* (1998) |
| 6R | 5'- GGA CCT TAC TCG TTT CCG ACC - 3' | |

To exclude amplification of sequences from other organisms caused by unspecific primer binding sites, the primers were tested with the BLAST algorithm[2] for homologies with other SSU rDNA-sequences in the database. Additionally, DNA was extracted from samples of invertebrates (mostly fly larvae), bacteria and protists scraped from the culture dishes and a pellet of the culture medium for specificity-testing with the bryo 18S-F/R-primers.
One 20 µL PCR reaction contained 10 µL 2x ReddyMix PCR Master Mix (ABGene), 0.35 µmol of each primer and 1 µL DNA. The mix was made up with PCR-grade water to 20 µL. Amplification conditions for both primer pairs are shown in Table 2.2.

**Table 2.2 PCR-programs for the primers used**

| | *bryo 18S-F/R* | | *5F/6R* | |
|---|---|---|---|---|
| initial denaturation | 95 °C – 4 min | 1 x | 95 °C – 5 min | 1 x |
| denaturation | 95 °C – 1 min | 35 x | 95 °C – 1 min | 35 x |
| annealing | 58 °C – 1 min | | 55 °C – 1 min | |
| elongation | 70 °C – 1:10 min | | 72 °C – 1 min | |
| final elongation | 70 °C – 5 min | 1 x | 72 °C – 5 min | 1 x |

To test for presence of DNA from *Tetracapsuloides bryosalmonae*, the samples were amplified with the 5F and 6R primer pair according to Kent *et al.* (1998) (Tab. 2.1). All PCR-reactions were conducted with an Eppendorf mastercycler.

[2]National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/BLAST

### 2.2.6 Histology and immunohistochemistry

The samples for light microscopy were fixed in 10% neutral buffered formalin, washed, dehydrated and embedded in paraffin by an embedding automat (Tissue-Tek VIP, Sakura Bayer Diagnostics). Sections of 5 µm thickness were cut with a 1140 Autocut-microtome (Reichert-Jung) and either stained with haematoxylin and eosin (H&E) or used for immuno-histochemistry. For the latter, the *Tetracapsuloides bryosalmonae*-specific, monoclonal antibodies P01 (Aquatic Diagnostics LTD) were used. The slides were dewaxed, hydrated and endogenous peroxidase activity was blocked in 3 % $H_2O_2$ in phosphate buffered saline (PBS, v/v) for 10 min. After washing with tris buffered saline (TBS), rings were marked around the sections with a Liquid Blocker Super PAP pen (Cedarlane®). Non-specific protein binding sites on the slides were blocked with normal goat serum (1:10 in TBS) for 10 min. After that, serum was poured off and 50 µL of the reconstituted P01 antibody (20 µg/mL in PBS) were pipetted onto the sections. The slides were incubated in a humid chamber for 60 min at room temperature. Subsequently, slides were washed 3 times with TBS and 100µL of goat anti-mouse polyclonal antibody (IgG) conjugated with horseradish peroxidase (ZYMED Laboratories; 3 µg/mL in TBS) were added to each section, followed by incubation in a humid chamber for 30 min. After washing, the areas where the antibody was bound to the tissue were visualized by 10 min incubation with a solution of 3,3-diaminobenzidine tetrahydrochloride (DAB) activated with $H_2O_2$. A brown precipitate is formed upon reaction of the DAB substrate with the peroxidase linked to the second antibody. The reaction was stopped by immersion of the slides in tap water and sections were counter-stained for 4 min with haematoxylin, dehydrated in an increasing ethanol series and mounted. Kidney samples of a PKD-positive and a PKD-negative rainbow trout were used as positive and negative controls for immunohistochemistry respectively.

## 2.3 Results

### 2.3.1 Bryozoan culture

With the methods described for culturing bryozoans, colonies of *Fredericella sultana* could be kept alive for up to 12 months. Particles of sediment were sticking to the surface of colony tubes of bryozoans obtained from the field, whereas the colonies grown in the lab were

transparent so that parasite stages could be observed easily (Fig. 2.2A). New colonies were raised success-fully from statoblasts. After transfer from +4 °C to room temperature (18 – 24 °C), colonies hatched after about 5 – 10 d (Fig. 2.2B + C) and started to colonize the Petri-dishes (Fig. 2.2D).

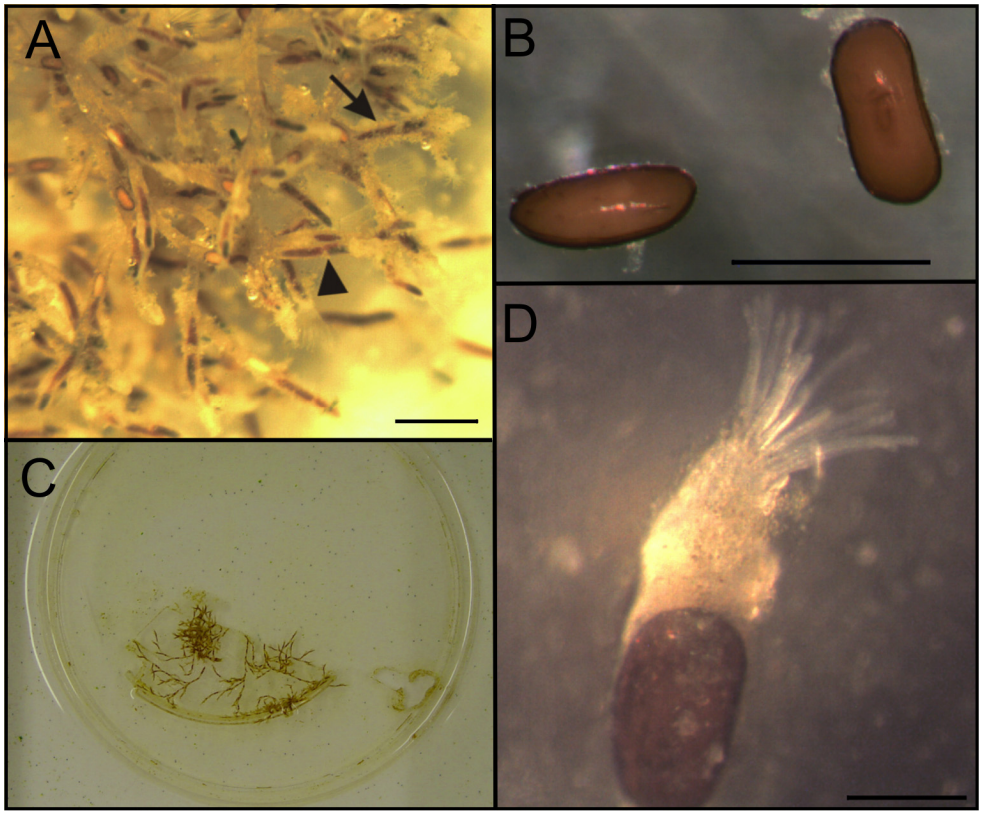


**Figure 2.2:** Cultured Bryozoa (*F. sultana*). **A**. Bryozoan colony. Accumulation of sediments (particles from mud in laboratory cultures) can be seen on some branches (arrow) while some are clear (arrowhead). Bar: 1 mm. **B**. Free statoblasts of *F. sultana*. Bar: 500 µm. **C**. Bryozoan laboratory culture on 12 cm Petri-dish. **D**. Newly hatched bryozoan zooid. Bar: 250 µm.

### 2.3.2 Test of statoblasts for cryptic parasite stages

Both the statoblasts from infected and uninfected bryozoan colonies were found negative for *Tetracapsuloides bryosalmonae* infection by PCR. The DNA extraction was shown to be successful with the protocol used. The amplification with the Bryozoa-specific 18S primer pair bryo 18S-F and bryo 18S-R resulted in the amplification of the expected 730 bp product for all statoblast samples and a part of a bryozoan colony used as positive control. No amplicon was obtained testing DNA from other organisms in the culture medium.

### 2.3.3 Fish infection

The kidney samples from brown trout, brook trout, rainbow trout and grayling tested by PCR 3 and 14 wpe to infected bryozoans were positive for *Tetracapsuloides bryosalmonae*. Therefore, it was assumed that the infection was successful and that the fish did not recover from the infection during the cohabitation trials. No external or internal PKD-related symptoms were observed in the fish dissected 3 wpe. Only rainbow trout showed clinical signs of PKD such as swelling of the body cavity and a slight exophthalmos from 5 wpe on. All the other fish species did not show any external clinical signs. Heavy internal signs of the disease, which were a highly swollen kidney and spleen, were detected in rainbow trout (Fig. 2.3) and grayling sampled 14 wpe, while brown trout and brook trout showed only a minor swelling of the kidney. Kidney samples of both bryozoan exposed pike, and non-exposed pike were tested negative by PCR.

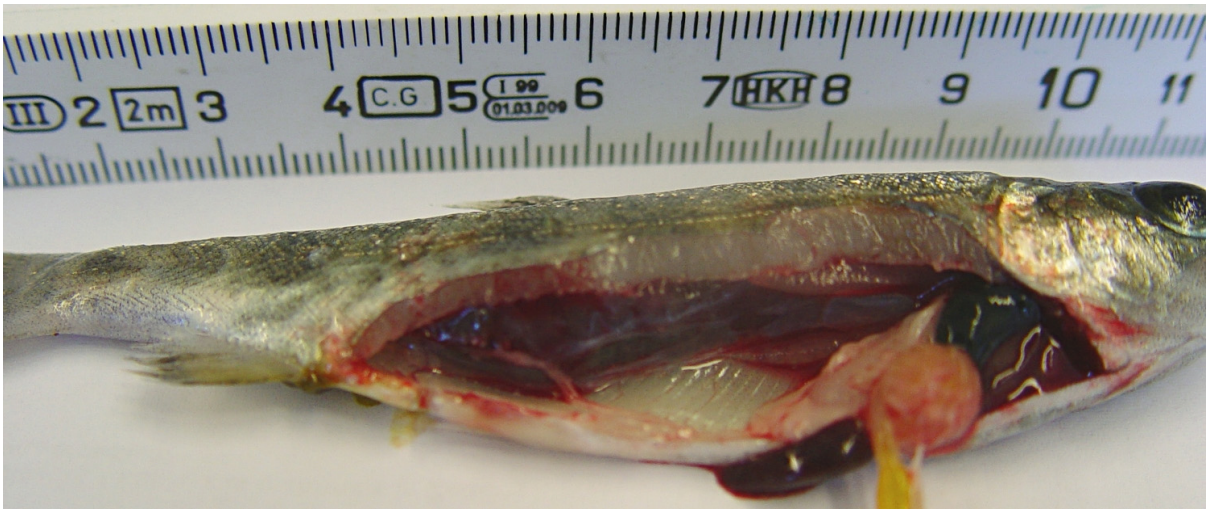


**Figure 2.3**: Infected rainbow trout 14 wpe showing clinical signs of PKD. Kidney is massively swollen, spleen is enlarged.

### 2.3.4 Histology and immunohistochemistry

Kidney sections of rainbow trout showed a high density of interstitial parasite stages and a proliferation of interstitial tissue (Fig. 2.4A). In grayling, the findings were similar but less pronounced (Fig. 2.4 B). Almost no proliferation of the kidney interstitium was observed in brook trout and brown trout in the kidney-sections investigated and the numbers of interstitial parasite stages detected by immunohistochemistry were low compared to those found in rainbow trout (Fig. 2.4C + D). Luminal stages could be detected with the *Tetracapsuloides*

*bryosalmonae*-specific P01-antibody in kidney sections of brown trout and rainbow trout (Fig. 2.4E + F), but not in those of brook trout and grayling. Maturing spores with polar capsules were exclusively observed in the lumen of kidney tubules of brown trout (Fig. 2.4F).

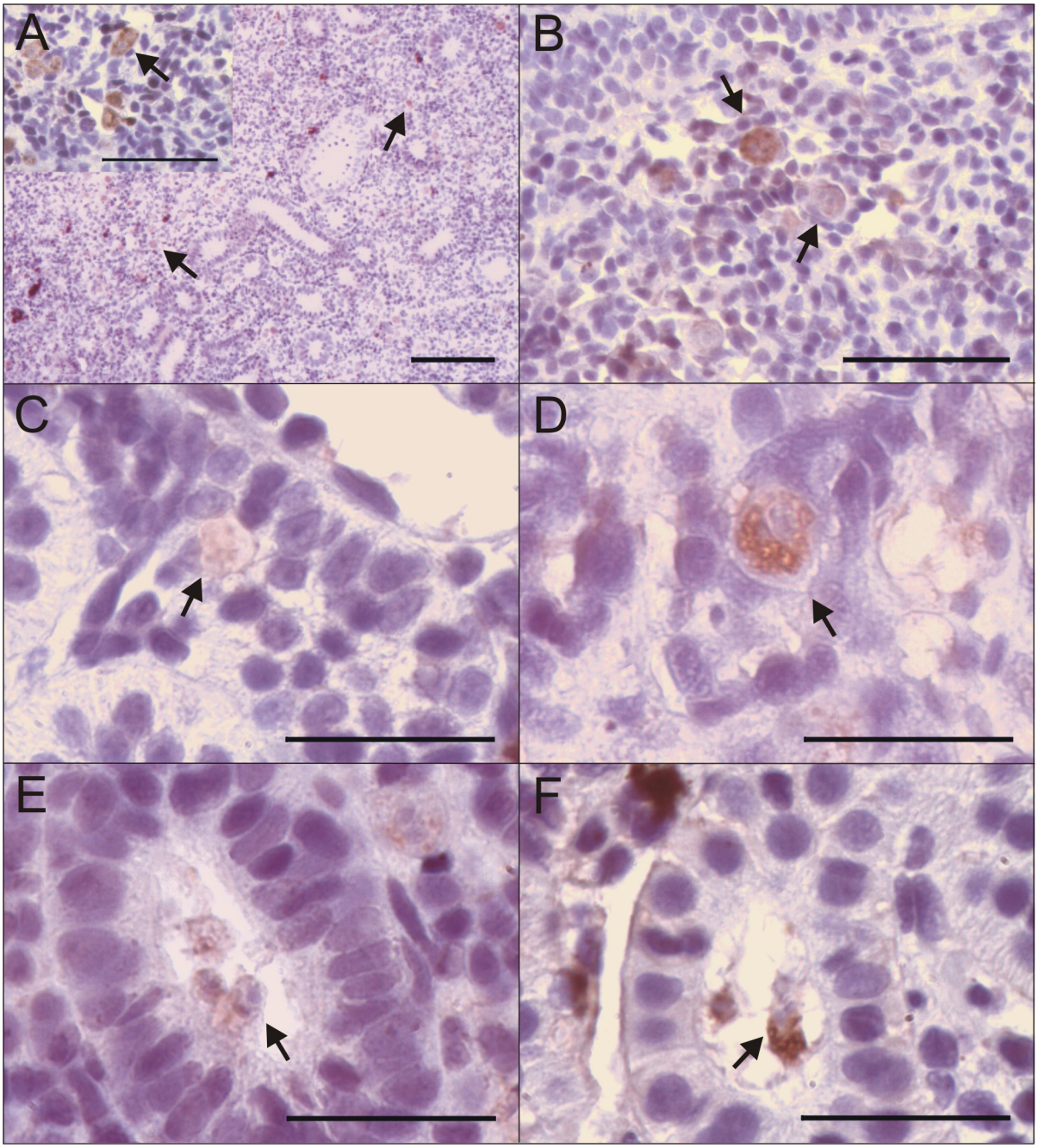


**Figure 2.4:** Kidney stages of *T. bryosalmonae* in the salmonids used in the present study. **A**. Low magnification of a rainbow trout kidney section. Numerous parasite stages (arrows) and proliferation of the interstitial tissue can be seen. Bar: 100 µm. Inset: Immunohistochemically stained parasites at higher magnification (arrow). Bar: 50 µm. **B**. Interstitial, two-cell stages (arrows) in a section of grayling kidney. Bar: 50 µm. **C**. Interstitial stage in brook trout kidney (arrow). Bar: 25 µm. **D**. Interstitial stage in brown trout kidney (arrow). Bar: 25 µm. **E**. Intraluminal stage in rainbow trout kidney (arrow). Bar: 25 µm. **F**. Intraluminal stage sporogonic stage in brown trout kidney. Polar capsules are visible (arrows).Bar: 25 µm. All sections were immunohistochemically stained with haematoxylin counter stain.

### 2.3.5 Infection of Bryozoa

Four wpe to infected brook trout, bryozoans showed typical *Tetracapsuloides bryosalmonae* sac-stages (McGurk *et al.* 2006a) in a small part of the colony (Fig. 2.5A+B). After 5 wpe, first aggregations of presaccular cells could be observed in the body cavity of bryozoans exposed to brown trout. Within the following three weeks more and more colony parts showed overt infections with maturing sac stages and spores floating in the metacoel. After 9 wpe, almost all bryozoan colonies that had been exposed to brown trout showed overt infections. The prevalence of infection in brook trout-exposed colonies was lower, but a direct comparison to the brown trout exposed bryozoans was impossible due to considerable differences in colony size. Bryozoa cohabitated with infected rainbow trout and grayling showed no visible *T. bryosalmonae* stages at any time up to the end of the experiment 6 months pe. No macroscopically visible parasite development was observed in unexposed control bryozoan colonies within the 13 months of observation.

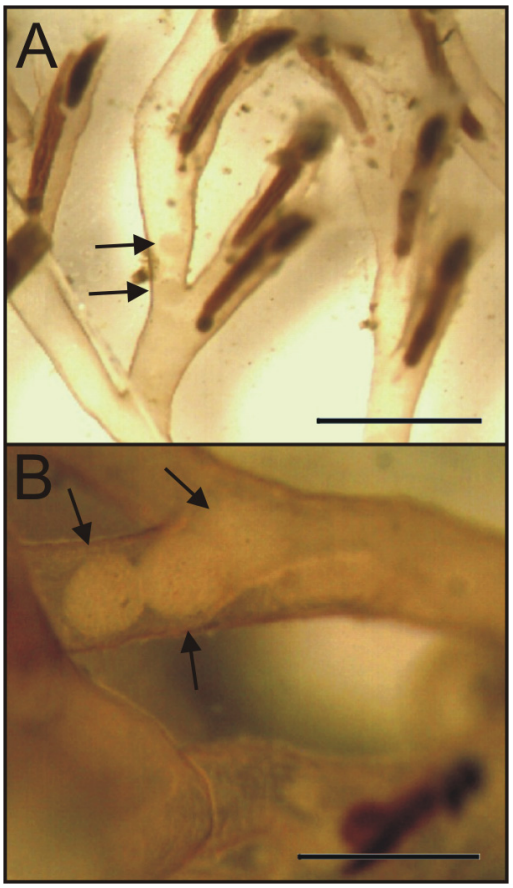


**Figure 2.5:** Sac-like stages of *T. bryosalmonae* in the bryozoan *F. sultana*. **A**. Infected *F. sultana* laboratory-raised colony. Two sac stages can be seen (arrows). Bar: 1000 µm. **B**. Higher magnification of infected colony. Three sac-stages visible (arrows). Bar 500 µm.

## 2.4 Discussion

Common culture methods for freshwater bryozoans are dependent on eutrophic aquaria and the presence of goldfish (*Carassius auratus*) in the same system with the bryozoans or the use of nutrient-rich pond water for culturing (Wood 1996, Tops *et al.* 2004). These methods are not useful if parasite-free bryozoan colonies are required for infection experiments, as both fish and pond water in the culture system might introduce unexpected infections. The use of pure algae as a food source for bryozoans has been applied previously (Oda 1980) and was

used already for studies of malacosporean infections in bryozoans (Morris *et al.* 2002a, McGurk *et al.* 2005b & 2006a + b). In the present study, bryozoans could be raised successfully from statoblasts and kept alive by feeding with different species of live algae for more than 12 months. This time was enough to perform infection experiments with *Tetracapsuloides bryosalmonae* and to keep the live-cycle in the laboratory continuously.

The possibility of transmission of cryptic PKD-stages via bryozoan statoblasts was already considered due to the apparently incomplete sporogenesis and the intense immune-response of fish hosts (see discussion in Tops *et al.* 2004). In the present study, no parasite DNA could be detected in *Fredericella sultana* statoblasts from infected colonies and no parasite stages could be observed in colonies raised from these statoblasts after more than one year. Nevertheless, it might be possible that dormant stages in bryozoan statoblasts may occur in some cases. For example, *T. bryosalmonae*-susceptible bryozoan species like *Plumatella repens* may incorporate stages of this parasite into their statoblasts more frequently than *F. sultana*. Another explanation for the lack of dormant stages in the statoblasts used in the present study could be that incorporation of stages into statoblasts is restricted to some strains of *F. sultana* or *T. bryosalmonae* and might reflect the adaptation to habitats with only few or no fish hosts. Taticchi *et al.* (2004) observed vermiform, motile objects, similar to stages of the malacosporean *Buddenbrockia plumatellae,* leaving from statoblasts of *Plumatella fungosa* that opened after KOH-treatment. In their work, it could not be clarified to which systematic group these worms belonged as no ultrastructural study or molecular was conducted, but this finding might be an evidence for malacosporean stages in bryozoan statoblasts. Additionally, DNA of a *Buddenbrockia* sp. was detected in 9 out of 10 statoblast-raised colonies of the bryozoan *Lophopus crystallinus* by PCR, although no overt infection was ever observed (Hill & Okamura 2007). These finding suggest that propagation of the parasite in statoblasts of the host might be a common strategy for some malacosporean species.

The laboratory infection experiments conducted in the present study demonstrated the ability of two fish species, brown trout and brook trout, to transmit *T. bryosalmonae* to *F. sultana*. In a previous study, PKD-infected brown trout were shown to transmit the parasite to *F. sultana* when the bryozoan culture was exposed to outflow from a tank with infected fish (Morris & Adams 2006a). The time course of the life-cycle in the study of Morris & Adams (2006a), beginning with the infection of the fish until the first stages were observable in bryozoan colonies, was similar to the observations of the present study. This shows that the life-cycle of

*T. bryosalmonae* can be reliably reproduced in the laboratory. A totally new finding of the present study was that PKD-infected brook trout were capable of infecting Bryozoa, and it can be assumed that this species could serve as a host for *T. bryosalmonae* under natural conditions. Arctic charr (*Salvelinus alpinus*) is known to be susceptible to PKD as well, and numerous intraluminal sporogonic stages of *T. bryosalmonae* could be detected in kidney tubules of this species (Kent *et al.* 2000). Obviously, other species of the genus *Salvelinus* might also be able to transmit PKD to bryozoans.

PKD also exists in North America. However, there are considerable genetic differences between isolates of *T. bryosalmonae* from North America and Europe, which led to the conclusion that the separation of the two strains must have substantially pre-dated fisheries activities and introduction of non-indigenous fish species to other continents by humans (Henderson & Okamura 2004). Because maturing spores of *T. bryosalmonae* were found in rainbow trout infected in North America, but not in rainbow trout infected in Europe (Kent & Hedrick 1986, Bucke *et al.* 1991), it was discussed that strain differences between parasites and/or hosts from the USA and Europe might exist (Morris *et al.* 1997, 2006a). This assumption was confirmed by the fact that rainbow trout infected with *T. bryosalmonae* were unable to infect bryozoans as shown by the present experiments. Hedrick *et al.* (2004) remarked that both, the number of maturing spores in the kidney tubules and the prevalence of spores in the urine of rainbow trout examined in their study were low and in one year, no spores could be observed at all. Therefore, it can be assumed that formation of spores is a rare event in rainbow trout. In the present study the infection of bryozoans with *T. bryosalmonae* may not have occurred because only three infected rainbow trout were used, which was not enough to infect the Bryozoa. It is not clear, if a European strain of this parasite exists that is able to complete its development in rainbow trout too. In a study conducted in Switzerland, Bettge *et al.* (2009a) measured the release of *T. bryosalmonae*-DNA from rainbow trout, but no attempts were made to show whether this DNA was derived from immature spores or just from cellular remains of parasite cells. Therefore it is not clear, if the fish used in these experiments would have been able to transmit the parasite to bryozoans.

It is typical for most myxozoans to cause comparatively mild symptoms in their natural hosts (Lom & Dyková 1992, Lom & Dyková 1995). Hence mild swelling of the kidney and the proliferation of interstitial tissue indicate that brown trout are the original hosts for *T. bryosalmonae* in Europe. In contrast, rainbow trout are less adapted, at least to the European strain

of the parasite, which is reflected by the occurrence of severe symptoms and high mortalities in these fish when infected with the European strain of *T. bryosalmonae* (Bucke *et al.* 1991).
The different host responses observed in the present study are unlikely to be attributed to different dosages of *T. bryosalmonae* spores, as the different species of fish, except grayling and pike, were infected at the same time together in one aquarium. Furthermore, it has been shown that even a very low number of spores per fish can cause heavy clinical signs of PKD in rainbow trout and that spore dose is not correlated to the severity of the disease (McGurk *et al.* 2006a).
The low abundance of interstitial stages in the kidneys of brook trout and brown trout might indicate that these fish were recovering from the disease. A similar observation was made by Clifton-Hadley & Feist (1989), who found few or no interstitial PKD-stages in brown trout 10 weeks or later after exposure to PKD-endemic water or intraperitoneal injection of developmental stages from homogenised kidney. In the same study intraluminal stages were found only in brown trout and not in rainbow trout. Intraluminal sporogonic stages can be present for a long time after the clinical signs of the infection had already disappeared (Kent & Hedrick 1986). In the experiments of the present study, this seemed to be the case for brown trout at least, where developing sporogonic stages with maturing polar capsules and only few interstitial parasites were seen in histological sections of kidney samples from 14 wpe. Feist & Bucke (1993) found no interstitial PKD-stages in experimentally infected brook trout, but in contrast to the present study, luminal stages were present in this species. The fact that no intraluminal parasitic stages were found in the kidney of brook trout may either be due to the low number of sporogonic stages in this species or it may indicate that the fish is in the process of recovery, so that the parasite stages have already been eliminated from the kidney tubules. Another explanation could be that only the single fish sampled for histological examination did not show any intraluminal parasitic stages. This could be due to individual variation in infection severity and progress. When fish infected with *T. bryosalmonae* were kept at water temperatures below 12 °C a significantly reduced parasite load was detected compared to fish kept at higher temperatures (Bettge *et al.* 2009b). Therefore, the low parasite load in the fish in the present study could not have been caused by the water temperature because the experiments were carried out at a temperature above 12° C.
The PKD-infected grayling used for the experiments of the present study could not transmit the infection to Bryozoa. These fish showed similar proliferation of kidney interstitial tissue as seen in to rainbow trout, which might indicate that the parasite is not adapted to this host.

Possibly, the parasite was somehow prevented from entering the kidney tubules efficiently, which led to an intensified multiplication of *T. bryosalmonae*-stages in the kidney interstitium. These results are contradictory to the findings of Bucke *et al.* (1991), Feist & Bucke (1993) and Morris *et al.* (2000a) who observed intratubular stages in PKD-infected grayling. It is possible that the parasite requires more time to form spores in grayling than was provided in the course of this experiment, yet bryozoans were cohabitated with the fish for six months and it is unlikely that formation of sporogonic-stages in grayling takes so much longer than in brown trout. Host and parasite strain differences, or the low sample size may also be responsible for the different findings in the present work compared to the results of previous studies. A constraint of this study was that none of the bryozoan colonies cohabitated with infected fish was tested by PCR at the end of the experiment for covert infections. However, it is unlikely that overt infections developed only in the colonies exposed to brown trout and brook trout, while infection remained in a covert state in bryozoans cohabitated with rainbow trout or grayling.

As northern pike did not become infected, or at least did not show any kidney stages after cohabitation with PKD-infected *F. sultana* colonies, it is doubtful that this species is susceptible to *T. bryosalmonae.* The myxozoan-like stages found in pike in previous studies (Seagrave *et al.* 1981, Bucke *et al.* 1991, Morris *et al.* 2000a) might be stages of other malacosporeans such as *Buddenbrockia* spp. Up to the present, there are two species described as parasites of bryozoans (Schröder 1910, Canning *et al.* 1996, Morris *et al.* 2002a, Canning *et al.* 2007), but no fish host has been identified yet for these malacosporeans. Morris *et al.* (2000a) used four monoclonal antibodies raised against *T. bryosalmonae* antigens and demonstrated a reaction with stages in pike kidney. This supports the hypothesis that the pike-parasite is closely related to Malacosporea. As pike in turn are considered to be closely related to salmonids (Nelson 1994), it is also possible that *T. bryosalmonae* adapted to some strains of pike in certain areas. Eszterbauer *et al.* (2006) discussed the speciation problem within the Myxozoa and suggested that different lineages of one myxozoan species with different spore-size or tissue specificity might develop, which can not be differentiated genetically by 18S rDNA-sequence comparison. Maybe such *T. bryosalmonae* lineages exist, one being adapted to trout and one to pike. A similar situation was reported from Canadian *Myxobolus arcticus* forming spores in sockeye salmon (*Oncorhynchus nerka*) (Kent *et al.* 1993) and Japanese *M. arcticus*, not being able to complete its development in this fish host (Yokoyama & Urawa 1997).

The present study showed that besides brown trout brook trout are also suitable hosts for *T. bryosalmonae*, while rainbow trout and grayling are not. In contrast to previous findings, pike could not be infected with *T. bryosalmonae*. Further investigations on different parasite strains from various geographical locations and their capability to infect different fish species are clearly warranted. These studies would help to clarify the entire host spectrum of *T. bryosalmonae* and different (genetic) adaptations of this parasite to the regionally available host species.

# 3. INVESTIGATIONS ON THE PORTAL OF ENTRY OF *TETRACAPSULOIDES BRYOSALMONAE* INTO THE FISH

## 3.1 Introduction

The development of *Tetracapsuloides bryosalmonae* in the kidney of the fish host has been described in detail by Ferguson & Needham (1978), Kent & Hedrick (1986), Clifton-Hadley *et al.* (1987) and Morris & Adams (2008), whereas the route of entry of the sporoplasm into the fish is widely unknown. Studying the invasion strategy of this parasite will help to understand the biology of this unique group of Bryozoa-infecting myxozoans. During the process of infection, the free floating actinospores of myxozoans are activated by chemical stimulants from the fish mucus and mechanically upon contact to fish surfaces like gills, skin or buccal cavity. Spores discharge their polar filaments that attach to the host. Subsequently, the sporoplasm leaves the spore shell and penetrates the epithelium of the host (Kallert *et al*. 2005, 2007). As the spores of *T. bryosalmonae* that are released from the bryozoan host (bryozoa-spores) have the same cellular organization like actinospores of myxosporeans (Fig. 3.1), a similar mode of infection can be postulated.

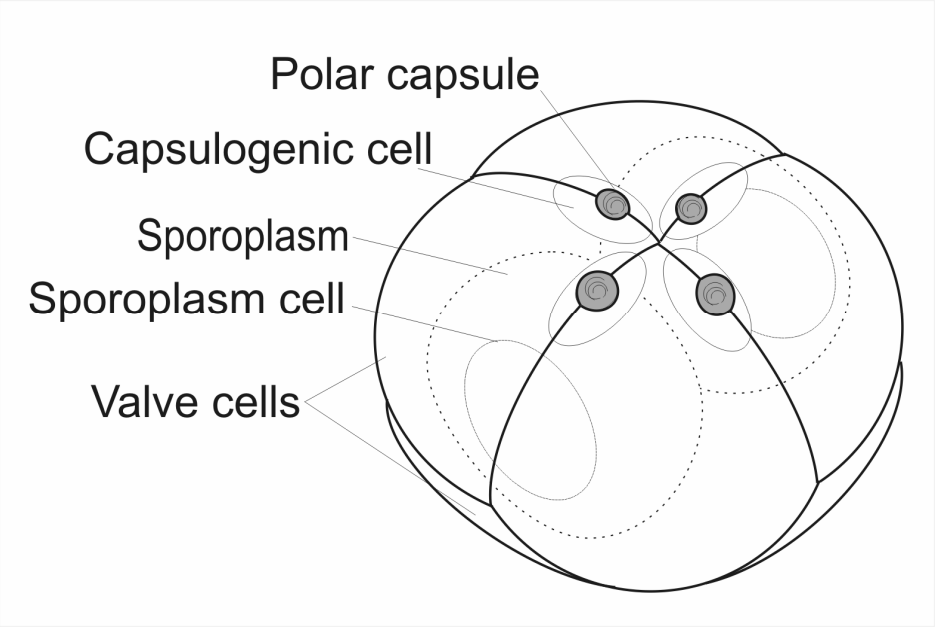


**Figure 3.1:** Schematic drawing of a *T. bryosalmonae* bryozoa-spore according to results of McGurk *et al*. 2005b and own observations. Nuclei of cells are not shown.

Results of previous studies on *T. bryosalmonae* indicate that the gills (Morris *et al.* 2000b) and epidermal mucus cells (Longshaw *et al.* 2002) are portals through which the parasite seems to enter the fish host. However, visualization of developmental stages of *T. bryosalmonae* in the previous studies did not provide satisfying information about the mode of penetration and the chronological progression of the early development. As the abundance of malacosporean-infected bryozoans is usually low (Anderson *et al.* 1999b, Okamura *et al.* 2001, Okamura & Wood 2002), the attachment to the host and the penetration process of these few spores must be achieved by an efficient mechanism. It could already be shown, that one single spore is sufficient to infect a fish and to cause clinical symptoms of PKD (McGurk et al. 2006a). In the present study, it was tested whether skin, gills or both organs are the main portals of entry for *T. bryosalmonae* into the fish and it was attempted to characterize the attachment and penetration process in detail. Stages, during and shortly after penetration were visualized by light and electron microscopy. To show the reaction of *T. bryosalmonae* spores that develop in bryozoans after contact to the fish host, *in vitro* activation experiments were conducted. Additionally, the bryozoa-spores were examined by scanning electron microscopy (SEM) for the first time.

## 3.2 Materials and Methods

### 3.2.1 Fish and infected Bryozoa

For the experiments, self-reared SPF rainbow trout (*Oncorhynchus mykiss*) fry (3 months post hatch, about 3 cm length) were used. The fish were kept in dechlorinated tap water at 10 - 12 °C until onset of the experiments.

To obtain adequate amounts of *Tetracapsuloides bryosalmonae* spores, laboratory infected colonies of *Fredericella sultana* with zooids containing mature spore sacs or free spores in the coelomic fluid were dissected (for laboratory culture methods of the bryozoans see section 2.2.2). For this purpose, overtly infected parts of the bryozoan colonies and spore sacs were torn apart with forceps and needles on a microscope slide. The spores obtained were washed with a small amount of tap water into a 5 mL glass vial and stored at +4 °C for less than 2 h until use. By counting aliquots of 20 µL of the spore suspension, the approximate spore number was determined.

### 3.2.2 Activation of spores

Rainbow trout mucus was obtained and purified according to Kallert *et al.* (2005). In brief, trout mucus was scraped off and was homogenized, centrifuged and the supernatant used for experimental activation. The homogenate was solubilised in distilled water and used at a final concentration of 2 mg/mL (dry weight). Spores were activated by a modified procedure according to Kallert *et al.* (2005) by pipetting 14 µL of spore suspension and 6 µL of trout mucus homogenate up and down three times. Subsequently, a cover slip was placed on the slide carefully without applying any pressure and the spores were investigated with a Zeiss phase contrast microscope.

### 3.2.3 Scanning electron microscopy (SEM)

Bryozoa-spores, whole fish and gills, incubated with 1,000 to 2,000 spores for 5 and 15 min, were prepared for SEM as follows. Mature spores were obtained as described above, while care was taken to minimize the amount of water for washing the spores from the slide (about 200 µL). For fixation, 2 % glutaraldehyde in phosphate buffer according to Soerensen (v/v, pH 7.4) was added to the spore suspension. After a minimum of 24 h fixation, the sample was centrifuged at 1000 rpm for 10 min in a Cytospin® 3 centrifuge (Shandon) to collect the spores on an L-lysin-coated slide. Whole fish and gills dissected from the fish after the respective incubation time were also fixed in 2 % glutaraldehyde in phosphate buffer. Both spores on the slides and gill/fish-samples were washed with phosphoric buffer for 2 h, and dehydrated in a graded acetone series (10 % 2.5 min, 20 % 2.5 min, 30 % 2.5 min, 40 % 2.5 min, 50 % 2.5 min, 60 % 2.5 min, 70 % 1 h, 80 % 1 h, 90 % 1 h, 95 % 1 h, 100 % over night; all v/v). The dehydrated sample was critical-point-dried (6 °C, 50 bar, 12 changes of medium; 41 °C, 98 bar, 15 min; pressure compensation over night). After that, the sample was sputtered with platinum (15 mA, 300 s, about 12 nm) and examined with a Zeiss digital scanning electron microscope 950.

### 3.2.4 Light and transmission electron microscopy (TEM)

For the histological examination of the penetration process on the surface and inside of the tissues, each rainbow trout was exposed to 1,000 to 2,000 spores in 100 mL of water at 15 °C.

This procedure was repeated with two fish at different exposure times of 5, 10, 20, 30 and 60 min respectively. Fish were killed by spinal severance after anesthetization and gills, pieces of the skin and fins were removed and fixed immediately for light and electron microscopy. Fixation and processing for light microscopy and immunohistochemical staining were already described in section 2.2.6. For electron microscopy, small pieces of tissues were fixed in 2.5 % glutaraldehyde in Soerensen phosphate buffer (v/v, pH 7.4) over night. Samples were then washed 3 times for 10 min in the same buffer to remove excess of fixative and to prevent hardening of the tissue or reaction with the post-fixative. Subsequently, samples were post-fixed in 1 % osmium tetroxide in Soerensen buffer (v/v) for 2 h and were washed again 3 times for 10 min in Soerensen buffer. Samples were dehydrated in a graded acetone series (70 %, 90 %, 95 % each for 20 min, 3x 100 % 10 min; all v/v) and transferred to epon resin through mixtures of 3:1, 1:1, 1:3 aceton:epon (10 min each). In pure epon, samples were left over night and were then transferred to gelatine capsules. These capsules were labelled and filled to the top with epon. After polymerization for 5 days at 50 °C, the gelatine was washed off and the blocks were trimmed. Semi and ultra-thin sections were cut on a Reichert-Jung Ultracut microtome. Semi-thin sections were stained with toluidine blue. Ultra thin sections were contrasted with 2.66 % lead citrate and 5 % uranyl acetate and viewed using a Zeiss EM 10.

## 3.3 Results

### 3.3.1 Activation of spores

After 1 to 5 min following activation, several bryozoa-spores opened at the side opposed to the polar capsules and released the sporoplasms. Immediately, the sporoplasm was seen to move slowly by formation of pseudopodia (Fig. 3.2). One sporoplasm remained in a position close to the remainders of the spore while the other slowly moved along a distance of about 50 µm until the slide dried out. Only the primary cell contributed to the movement. The movie sequence on the supplementary DVD shows the recorded movement of the sporoplasms shown in figure 3.2 in real-time (arrows point to forming pseudopodia).

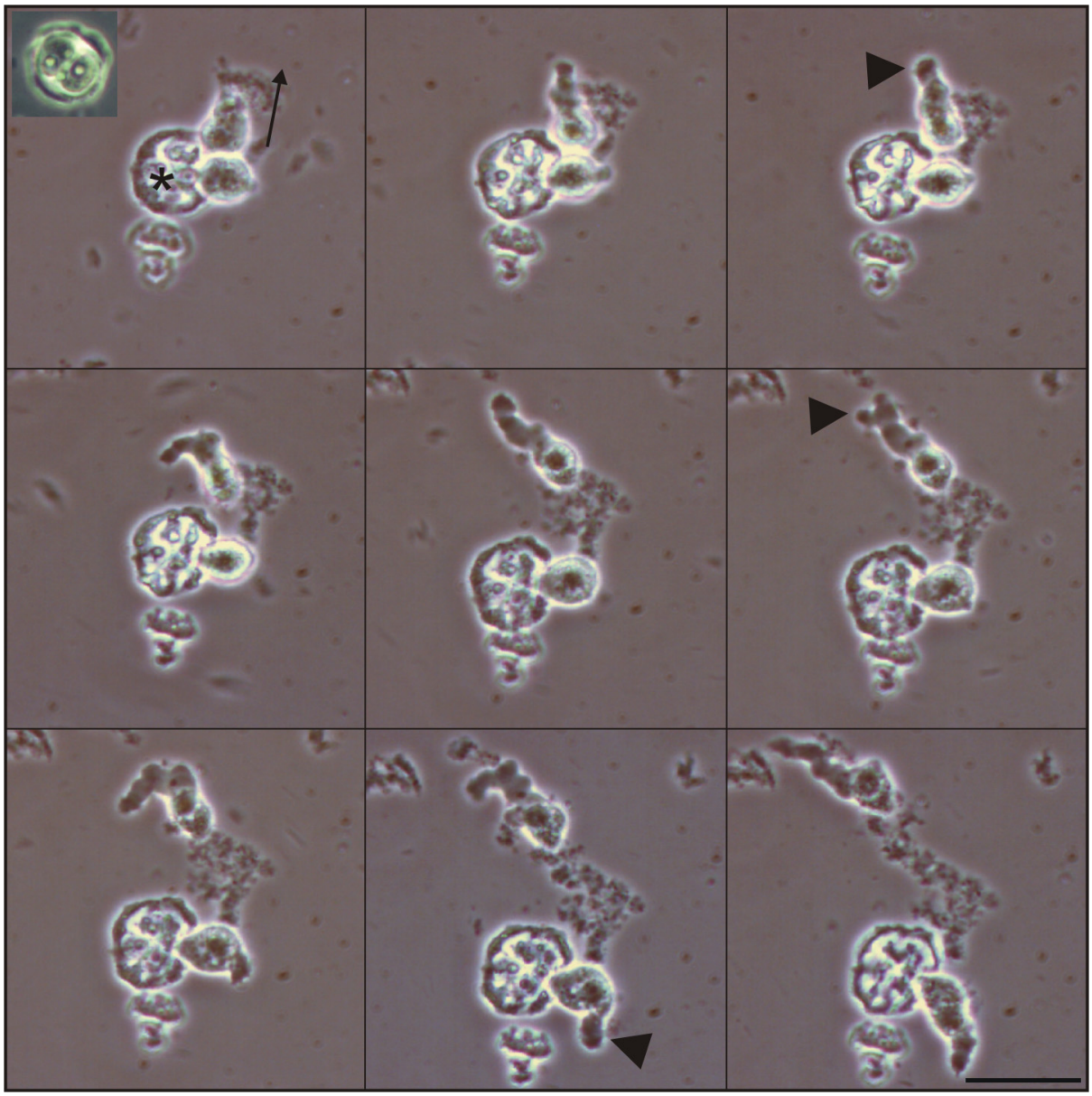

**Figure 3.2:** Time-sequence of bryozoa-spore of *T. bryosalmonae,* activated by addition of trout mucus homogenate and by pipetting up and down (start: top left, end: bottom right). Inset in top left corner shows non-activated spore for comparison. Sporoplasms leave the spore and one starts moving upwards (arrow). Formation of pseudopodia can be observed in both cells (arrowheads). Remainder of spore (valve cell and capsulogenic cells with polar capsules) are marked by an asterisk. Bar: 25 µm.

In other cases, sporoplasms left the spore but did not show further movement after contact to water, although formation of pseudopodia could be observed (Fig. 3.3). Surprisingly, no polar filaments were seen fired by the activated spores, even though the sporoplasms were released.

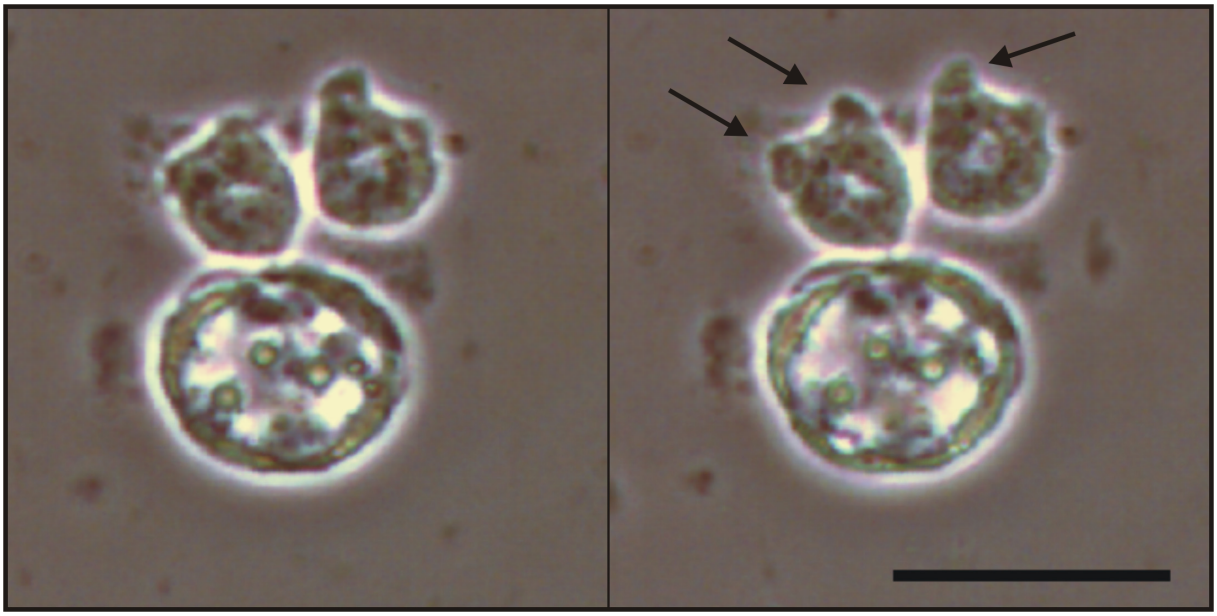

**Figure 3.3:** Bryozoa-spore of *T. bryosalmonae,* activated by addition of trout mucus homogenate and pipetting up and down. Sporoplasms leave the spore and form pseudopodia (arrow). Bar 25 µm.

### 3.3.2 Scanning electron microscopy (SEM)

Spores of *Tetracapsuloides bryosalmonae* could be successfully prepared for SEM, although the final spore number on the slide was low due to high losses during processing. Figure 3.4A shows a top view of a bryozoa-spore. Four visible valve cells almost completely covered the polar capsules. Polar filaments were fired in some spores (Fig. 3.4B), probably due to denaturation of integral components by the fixative. Only a small opening for the polar filament remained along the suture-line of two valve cells (Fig. 3.4B+C), which was sealed by a plug-like insertion (Fig. 3.4D). One sporoplasm could be seen leaving the spore at the bottom side, showing pseudopodial processes. Surprisingly, no spores could be found on the gills or on the body surface of *T. bryosalmonae*-exposed trout.

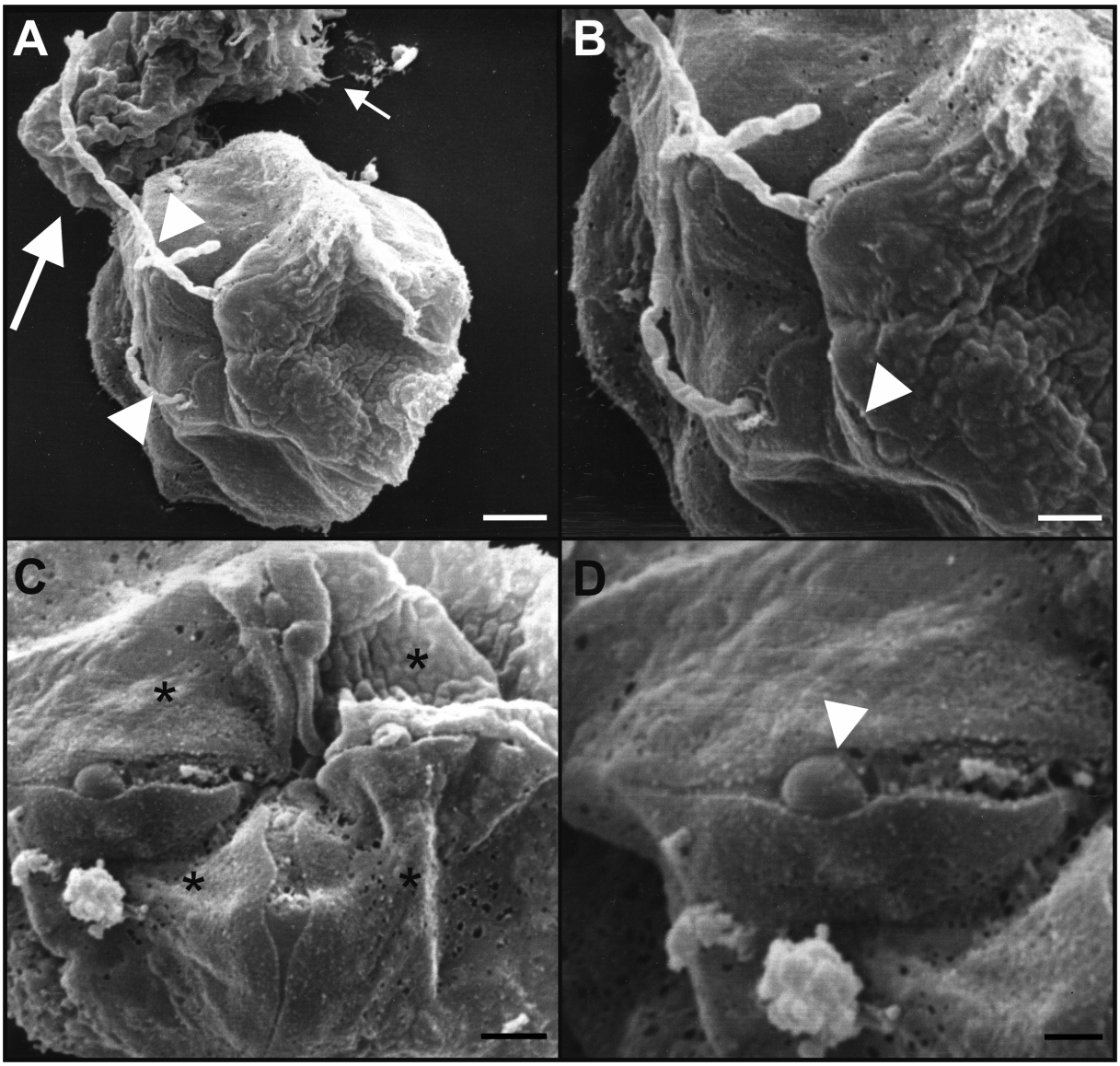


**Figure 3.4:** SEM pictures of *T. bryosalmonae* bryozoa-spores obtained from dissected *F. sultana*. **A**. Top view of a *T. bryosalmonae*-spore with its four polar capsules covered by valve cells. Two polar filaments are fired (arrowheads). Sporoplasm is leaving the spore at the bottom side (arrows). Formation of pseudopodia can be seen (small arrow). Bar: 2 µm. **B**. Higher magnification of A. Suture lines (arrowhead) of two valve cells. Bar: 1 µm. **C**. Apical view of polar capsule region. Plugs sealing the openings of the polar capsules between the 4 valve cells (asterisks). Bar: 1µm. **D**. Higher magnification of C showing plug (arrowhead). Bar: 500 nm.

### 3.3.3 Light and transmission electron microscopy (TEM)

In light microscopy of immunohistochemically stained paraffin sections, spores attached to the gills were seen at all time points, with the highest number of spores (1 – 4 per section of a total gill arch) observed at 20 min post exposure (pe) (Fig.3.5A). Some spores had the sporoplasm still enclosed by the valve cells (Fig. 3.5B), but sometimes the original shape of the

spore was lost and the spore wall opened to release the sporoplasms (Fig. 3.5C). In most cases, the gill epithelium was disrupted in the area of contact to the spore (Fig. 3.5B+D).

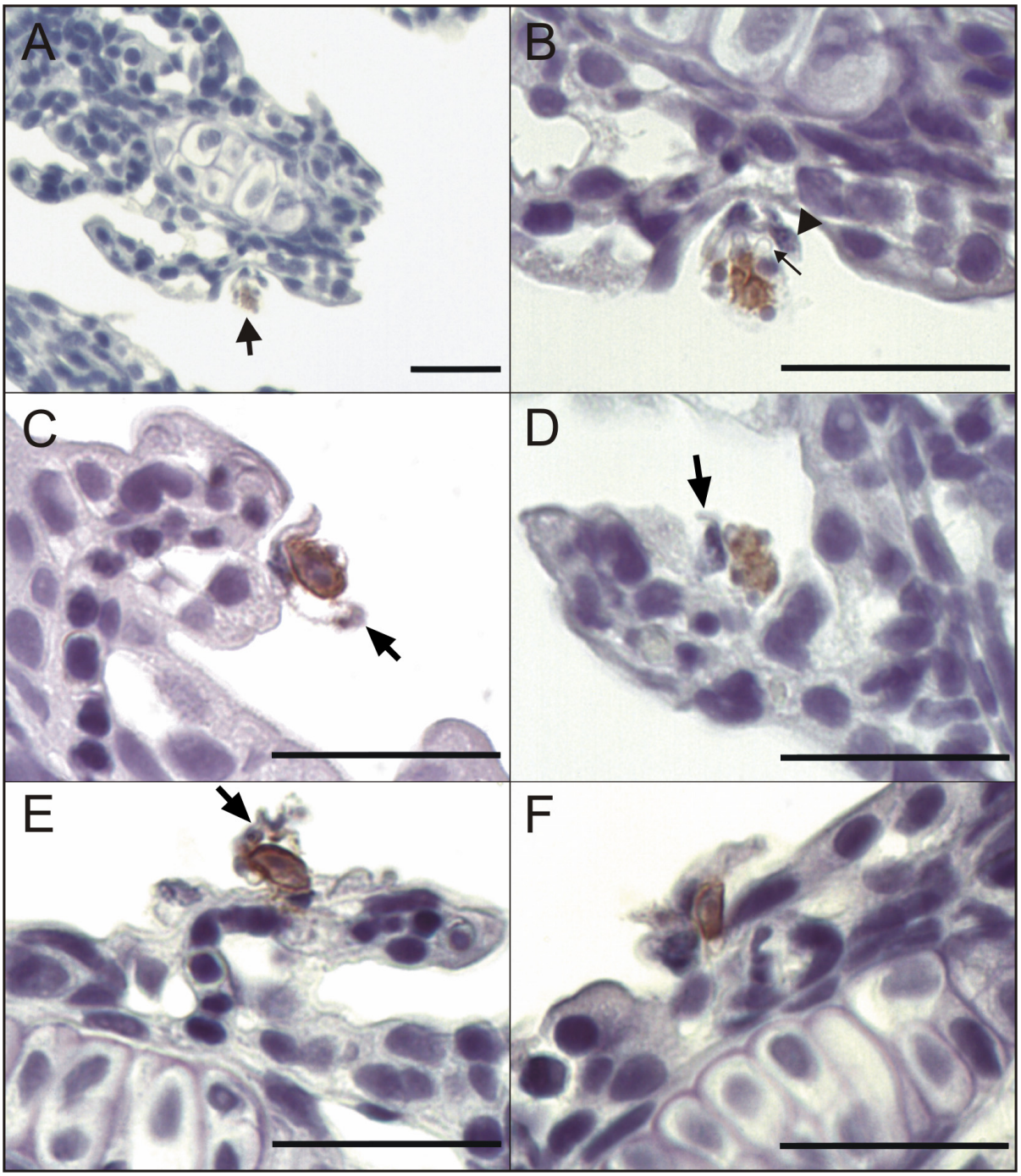


**Figure 3.5:** Light microscopy of gill sections of *T. bryosalmonae*-exposed rainbow trout. Spores attached to the gill. **A**. Spore attached to gill 20 min pe (arrow). **B**. Higher magnification of A. Sporoplasm stained brown surrounded by unstained valve cells. 2 polar capsules are in plane of section (arrow). Gill epithelium impaired in the area of contact to the spore (arrowhead). **C**. Sporoplasm 5 min pe in contact with gill. Remainders of valve cells detectable (arrow) **D**. Spore attached to gill 20 min pe. Valve cells still intact. Disrupted gill epithelial cell visible (arrow). **E**. Sporoplasm during penetration 30 min pe. Remainders of valve cells visible (arrow). **F**. Sporoplasm penetrating gill epithelium 30 min pe. All Figures: Immunohistochemical staining with haematoxylin counter stain, parasite sporoplasms stained brown. Bar: 25 µm.

After 30 min pe numerous parasite cells were observed to penetrate the gill epithelium (Fig. 3.5E+F). The earliest time point when sporoplasms could be detected inside of the gill tissue was 20 min pe (Fig. 3.6A+B). Possibly, these intraepithelial stages were heading for blood vessels, as parasite cells were seen in close contact to pillar cells after 60 min pe (Fig. 3.6C). In one section a parasite stage was detected in deeper layers of the gill (Fig. 3.6D), but it could not be differentiated if this penetrated stage consisted of one or two cells. No parasites were observed in deeper layers of the gill arches or inside of blood vessels.

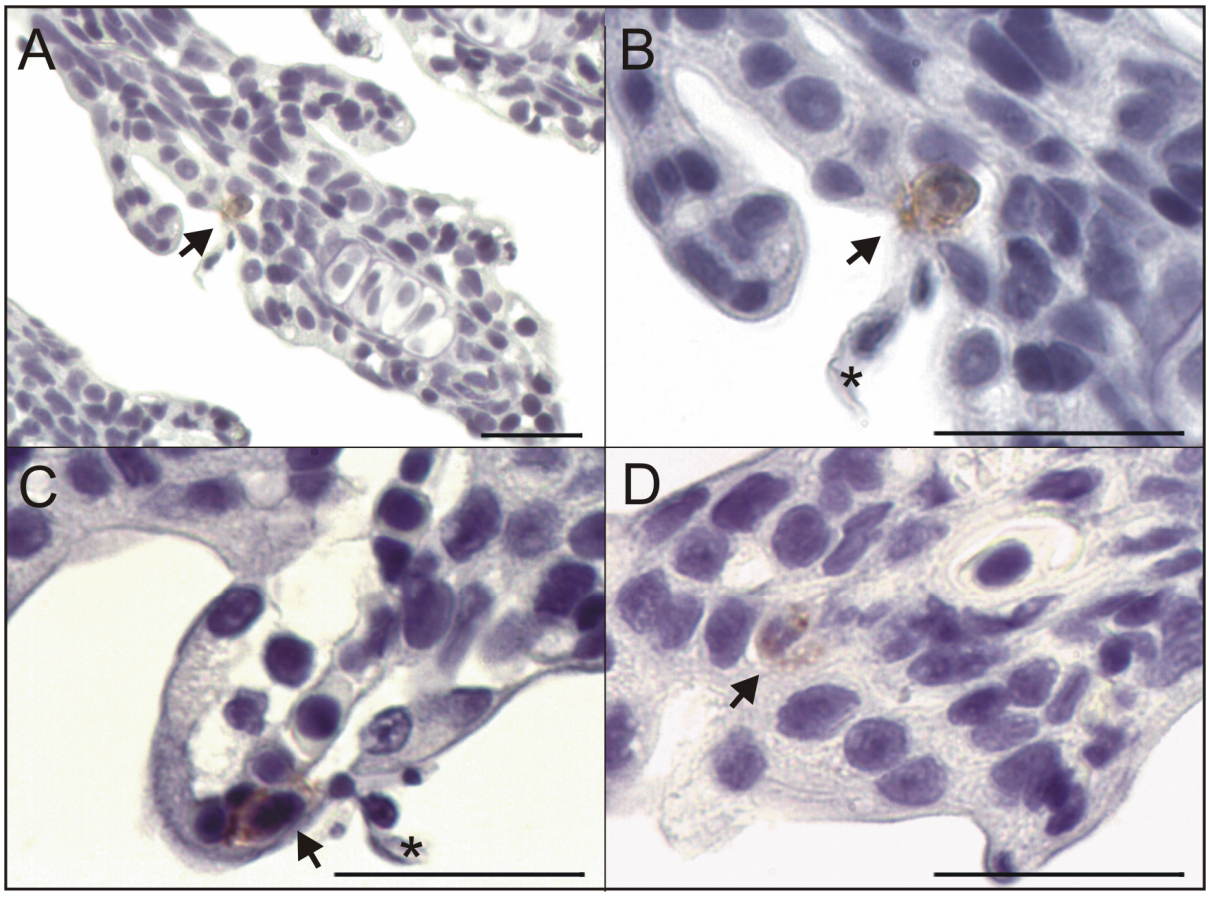


**Figure 3.6:** Light microscopy of *T. bryosalmonae*-exposed rainbow trout gill sections with penetrated sporoplasms. **A**. Sporoplasm in gill epithelium (arrow), 20 min p.e. **B**. Higher magnification of A. Parasitic cell between epithelial cells (arrow). **C**. Sporoplasm underneath gill epithelium (60 min pe), close to pillar cell. **D**. Parasitic cell in deeper layer of gill tissue (arrow) 60 min pe. All Figures: Immunohistochemical staining with haematoxylin counter stain, parasite sporoplasms stained brown. Asterisk denotes disrupted gill epithelium. Bar: 25 µm.

As the spore density on the gills was low, only a few parasite stages were found 20 min pe in ultra thin sections in TEM. Similar to the observations made by light microscopy, some spores attached to the gill epithelium were still intact with sporoplasms enclosed by the valves (Fig. 3.7A+B). The degeneration of the gill epithelium in the area of contact to the spore was

also observed in TEM (Fig. 3.7A). Penetrated stages in the tissue of the gills were not identified. No parasite stages were observed in the immunohistochemically stained paraffin sections of skin and fins, therefore these samples were not examined by TEM.

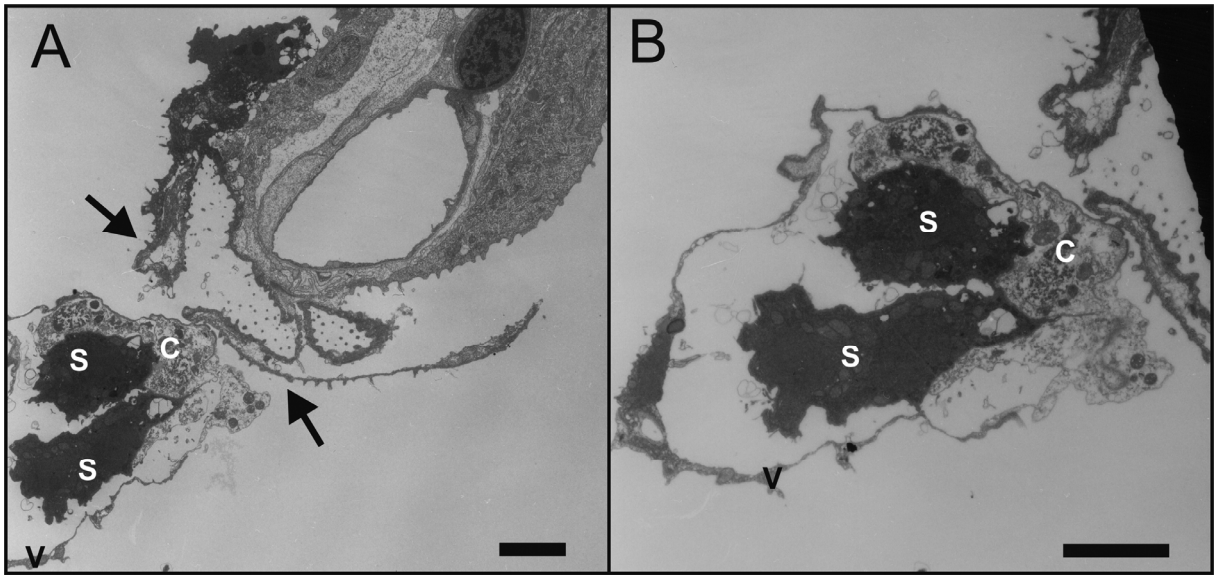


**Figure 3.7:** Transmission electron microscopy of the gill of *T. bryosalmonae*-exposed rainbow trout. **A**. Spore adjacent to the gill epithelium. Impairment of gill epithelium visible (arrows). Bar: 3 µm. **B**. Higher magnification of A. Sporoplasms are still enclosed by valve cells. Bar: 3 µm. V: valve cell, C: capsulogenic cell, S: sporoplasm.

## 3.4 Discussion

The results of the present study show that spores of *Tetracapsuloides bryosalmonae* obtained from infected bryozoans seem to target mainly the gill as portal of entry into the fish host. These findings are in conformity with the results of Morris *et al.* (2000b), who detected *T. bryosalmonae* stages in gill arches of rainbow trout 3 days after exposure to PKD-endemic waters by *in situ* hybridization. The authors found no other tissues to be infected at this time point. The stained cells they detected did not contain secondary cells and were therefore different from typical extrasporogonic cell doublets. Holzer *et al.* (2006) also demonstrated the presence of *T. bryosalmonae*-stages in blood vessels of the gills in farmed brown trout (*Salmo trutta*) and suggested this organ as the main route of entry for this parasite. In contrast to these findings, Longshaw *et al.* (2002) detected *T. bryosalmonae*-stages by *in situ*-hybridisation mostly in, or adjacent to skin mucus cells of rainbow trout exposed for only 1 min to a homogenate of infected bryozoans. Also at all other time points (1 to 90 min pe), the highest number of parasite stages was present in the skin and gill-stages were only found

in fish exposed for 90 min and 72 h to the bryozoan homogenate. According to the results of the present study, it can not be ruled out completely that *T. bryosalmonae* might also be able to penetrate through the skin, but the absence of stages in histological sections of this organ indicates that this is at least rare.

The process of attachment and penetration to the fish host was described thoroughly for the myxozoan *Myxobolus cerebralis*. This parasite was found to penetrate through the openings of mucus cells of gills, skin, and buccal cavity of rainbow trout (El-Matbouli *et al.* 1999). *M. cerebralis* first multiplies in the epidermal cells before it migrates through the peripheral nerves and the central nervous system to the cartilage, where spores are formed (El-Matbouli *et al.* 1995). In contrast, *T. bryosalmonae* is transported to the target tissue by the blood stream, and no multiplication step could yet be proven prior to blood/kidney stages (Morris *et al*. 2000b, Holzer *et al*. 2006). According to the short period (detectable by histology after about 1 week) required for the parasite to reach the target tissue (Morris *et al*. 2000b) it can be assumed that penetrated parasite stages directly migrate towards blood vessels and infiltrate kidney tissue. The gill epithelium is the thinnest barrier between the surrounding water and the blood stream, and therefore an ideal portal of entry for *T. bryosalmonae*. The contradictory findings of Longshaw *et al.* (2002) that the skin is the preferred site of entry to the fish for *T. bryosalmonae* could not be confirmed herein. Penetration through the skin might occur occasionally, but will presumably not lead to successful infection of the fish. This was observed for penetrating stages of the aurantiactinomyxon-spores of the gill-specific myxozoan *Henneguya ictaluri*, which were always found in the gills, but also in skin and buccal cavity after 24 h pe of the fish to spores of the parasite. Only the gill-stages developed further, while stages in all other organs degenerated after 96 h pe (Belem & Pote 2001). It can be speculated that the same is the case for *T. bryosalmonae*, but this does not explain why no attached spores and no penetrated sporoplasms were found in the skin in the study of Morris et al. (2000b) and in the present study. Additionally, some evidence indicates that *T. bryosalmonae* preferentially enters through the gills. Spores of *T. bryosalmonae* released by the bryozoans are very small compared to other myxozoan actinospores. It was stated previously that in most cases smaller spore types seem to be adapted rather to enter the gills than larger ones (Yokoyama & Urawa 1997). Like for *H. ictaluri*, it was found that the aurantiactinomyxon spores of *Thelohanellus hovorkai* are well adapted to penetrate the gills (Yokoyama & Urawa 1997). Also, the portal of entry of the echinactinomyxon spores of *Sphaerospora truttae* was located in the mucus cells of the gill, and to a lesser extent in skin and fins (Holzer *et al.*

2003). In contrast, the larger raabeia spores of *Myxobolus cultus* were found to penetrate the skin, fins but not gills and also the triactinomyxon spores of *Myxobolus arcticus* enter through the skin, fins, and rarely through the gills (Yokoyama & Urawa 1997). Possibly the smaller size of spores leads to an increased uptake by the fish and therefore the likelihood is higher to come in contact with the gills than with the skin.

Furthermore, according to the histological examination in the present study, the actual penetration of the sporoplasm into the gills took quite a long time. The first sporoplasms penetrating the gill epithelium were found 30 min pe. Before this time, only intact spores adjacent to the gill, where sporoplasms were still enclosed by valve cells, were detected in histological sections. Mechanisms and stimuli that induce actinospores released by the invertebrate host to become activated, to fire their polar filaments and to release the sporoplasm have been investigated in previous studies (Kallert *et al.* 2005, Yokoyama *et al.* 2006, Kallert *et al.* 2007). Thereby, differences in reaction time after chemical or a combination of chemical and mechanical stimulation were noted between actinospores of various myxozoan species. *T. hovorkai* aurantiactinomyxon spores and *Myxobolus parviformis* triactinomyxon spores needed a longer time to release their sporoplasms than triactinomyxon spores of *M. arcticus* and the triactinomyxon spores of *M. cerebralis* (Yokoyama *et al.* 2006, Kallert *et al.* 2007). The first two species are gill-specific and also show some morphological similarities. Their spores are rather small and the tips of the polar capsules do not significantly protrude above the apical region of the spore. The latter two species penetrate via various tissues, mostly the skin and have large spores with protruding polar capsule tips. As discussed before by Kallert *et al.* (2007), these morphological and behavioural characteristics are likely to be specializations to the respective portal of entry. The spores of *T. bryosalmonae* are also small and SEM observations show polar capsules that are almost entirely covered by the valve cells. In this context it has to be noted that no polar filaments were fired by mucus-activated spores of *T. bryosalmonae*, even though sporoplasms were leaving the spore and starting to move. This was also the case for activated spores of *M. parviformis* (Kallert *et al.* 2007), indicating that additional or different stimuli are required to induce the release of the polar filament. The excretion of various ions through the gill that will not be present in the mucous layer of the skin in the required concentration to trigger the polar capsules might be responsible for the observed preference of some species for this organ.

These findings show that to some extent conclusions can be drawn from size and morphology of the spore to the site of entry and further substantiate that the gill is the preferred portal of

entry for the spores of *T. bryosalmonae*. In this respect, it has to be noted, that the bryozoa-spores of *T. bryosalmonae* are spherical and do not have a style and caudal projections. Nothing is known about the functional morphology of different actinospore-types, but lack of caudal projections indicates that the bryospores sink faster than other actinospores. This is in compliance with the short life-span of these spores (de Kinkelin *et al*. 2002). It can be assumed that the contact to the fish host must occur short after release of the spores by bryozoans.

After attachment of a bryozoa-spore to the fish host, most of the spore "body" is discarded and only the sporoplasm remains that penetrates the tissue. This process was observed already for other myxozoans. For example the amoeboid movement of the sporoplasm by formation of pseudopodia was observed for *M. cerebralis* in histological sections and in sporoplasms released from activated spores (Markiw 1989, El-Matbouli *et al.* 1995, Eszterbauer *et al.* 2009). The observation of activated spores on a glass slide in the present study showed for the first time that sporoplasms of *T. bryosalmonae* move in an amoeboid fashion upon attachment to the host. In contrast to *M. cerebralis* that was found to penetrate mostly through the openings of the mucus cells (El-Matbouli *et al.* 1999), spores of *T. bryosalmonae* seemed to impair the gill epithelium upon attachment. The invasion of sporoplasms seemed to occur through the gill epithelium and not the mucus cells. Morris *et al*. (2000b) observed only single cells of *T. bryosalmonae*, not cell doublets in the gill tissue. This indicates that the primary cell of the sporoplasm is needed only for transportation of the secondary cell into the host tissue, like in all other actinospores. No indication for a release of the sporoplasm cell from the primary sporoplasm could be found in the present study. Either this takes place later in the course of infection or this process was not captured in the sections examined.

McGurk *et al.* (2005b) investigated spores of *T. bryosalmonae* using confocal laser scanning microscopy and found 8 valve cells, 4 on the upper half of the spore covering the capsulogenic cells and 4 on the lower half. In the SEM observations of *T. bryosalmonae* in the present study, only the four upper cells were seen, as only the apical side of the spores was visible. The reason for this effect is not clear. Probably there is a preference for this "upside-up" orientation of the spores during the cytospin preparation of the SEM-samples. It was also observed that the suture line of two valve cells was located over the polar capsules and left an opening for the polar filament. The umbrella-like plug, characteristic for malacosporean spores described previously in electron microscopical studies (Canning *et al.* 2002, Morris & Adams 2007a) was also visible from the outside of the spore in SEM. It was not obvious how

this plug is removed when the polar filament is fired. No conclusion could be drawn from the comparison of polar capsule-openings with and without released polar filaments. The lack of parasite stages on the gills and on whole fish prepared for SEM may be explained by a loss of these stages during processing of the samples. Improvement and optimisation of the processing procedure are required to be able to investigate the attachment and penetration process of *T. bryosalmonae* by SEM.

In conclusion, the present study provided further evidence for the hypothesis, that the gill is the preferred portal of entry for *T. bryosalmonae* to the fish host. Penetrating stages of this parasite on the gill could be visualized by light microscopy for the first time. Possibly, certain morphological features of myxozoan spores released by the invertebrate host allow generalizations about the entry locus into the fish. The low spore numbers on the gills hampered detailed TEM-studies of penetrating stages inside of the gill tissue. Further studies with higher spore numbers and longer exposure times are required to characterize the morphology of the early stages of *T. bryosalmonae* in the fish host and to allow conclusions about the fate of sporoplasms penetrating the skin.

# 4. SUSCEPTIBILITY OF DIFFERENT RAINBOW TROUT STRAINS TO *TETRACAPSULOIDES BRYOSALMONAE*

## 4.1 Introduction

Various diagnostic methods for *Tetracapsuloides bryosalmonae*, besides conventional histology and tissue impressions, have been developed. For molecular diagnostics polymerase chain reaction (PCR) is most commonly used and a reliable tool to detect even low grade infections by the parasite (Saulnier & de Kinkelin 1997, Kent *et al.* 1998, Morris *et al.* 2002b). Additionally, a loop-mediated isothermal amplification (LAMP)-test for *T. bryosalmonae* was developed recently (El-Matbouli & Soliman 2005). For the detection of parasite stages in histological sections of fish tissues various methods are available, including lectin histochemistry (de Mateo *et al.* 1993), monoclonal antibodies (Adams *et al.* 1992, Saulnier & de Kinkelin 1996, Morris *et al.* 1997) and *in situ* hybridisation (Morris *et al.* 1999). Quantitative real-time PCR (qRT-PCR) is a comparatively new method for diagnosis, and also accurate quantification of parasite stages (measured as parasite DNA) in the host.

As it was stated in the general introduction, up to now, no practicable treatment is available for PKD. Therefore, the use of trout strains resistant to *T. bryosalmonae* seems to be a possibility to overcome the problems associated with PKD in commercial trout farms. In a previous study, no difference in susceptibility was found between two rainbow trout (*Oncorhynchus mykiss*) strains to *T. bryosalmonae* (El-Matbouli *et al.* 2009), though it was known that these strains differed considerably in susceptibility to *Myxobolus cerebralis*, the causative agent of Whirling Disease (Hedrick *et al.* 2003). Thus, the aim of the present study was to collect more data about possible strain and species differences regarding susceptibility to *T. bryosalmonae* experimentally and systematically. Four rainbow trout strains and brown trout (*Salmo trutta*) were infected with *T. bryosalmonae* and parasite load during the preclinical phase of infection was assessed by means of qRT-PCR and immunohistochemistry. To this end, primer for a new qRT-PCR-assay based on Sybr-Green detection were developed and optimised.

## 4.2 Materials and Methods

### 4.2.1 Fish infection

For this experiment 50 fish each of four rainbow trout strains (DG, 16 months old; TL, 8 months old; SB, 9 months old; WT, 8 months old) and brown trout (BT, 6 months old) were used. TL is a North American trout strain with known susceptibility to the myxozoan *Myxobolus cerebralis* (Hedrick *et al.* 2003), causing Whirling Disease in salmonid fish. The WT fish were the offspring of a wild rainbow trout population in Germany. For the WT strain a considerably higher resistance to *M. cerebralis* was found compared to TL (El-Matbouli, unpublished results). DG and SB are rainbow trout strains cultivated in Germany. To assess possible susceptibility differences between different trout species, BT were included in this study. The fish of TL and WT strains were raised from eggs under specific pathogen free (SPF) conditions in our institute. Juvenile SPF SB and DG rainbow trout and BT were raised from eggs in a local fish hatchery, where they were kept in plastic tanks fed with spring water. Before the experiment, all fish of each strain/species were kept separately in 200 L aquaria with flow through of dechlorinated tap water for 2 months.

Originally, a laboratory infection was intended, but the spore-production of the PKD-laboratory-cycle was too low to infect the required number of fish. Therefore, the fish were exposed in late summer for 5 days to PKD spores in the outlet of a fish farm with known PKD history (El-Matbouli & Hoffmann 2002). Fish were kept in 302.5 L nylon cages (100 cm x 55 cm x 55 cm) with a mesh size of 2 mm. The water temperature in the hatchery was around 15 °C. After 5 days exposure, the fish were transferred back to the laboratory and kept in 200 L aquaria with flow through water at a mean temperature of 14.8 °C varying between 14.4 °C and 15.4 °C. The aquaria were checked daily for mortality or extraordinary behaviour. Dead fish were removed immediately from the aquaria, checked for ectoparasites or disease signs and organs were examined by standard bacteriology and histology. After transfer to the laboratory low-grade infections with *Trichodina* sp. and *Ichthyobodo necator*, were detected on all fish. To avoid excessive multiplication of the ectoparasites, fish were treated after 14 d post exposure with 1% NaCl (w/v) for 1h.

Eight fish from each group were sampled at 2, 3 and 4 week post-exposure (wpe) for detection and quantification of *Tetracapsuloides bryosalmonae*. Fish were killed by an overdose of MS 222 anaesthetic (Sigma-Aldrich), were weighed, measured (body length), checked for

skin and gill ectoparasites and overall appearance of the inner organs was assessed. A 10 - 40 mg piece of the kidney below the dorsal fin was removed and frozen at -20 °C until DNA extraction. This part of the kidney was chosen to allow reproducible sampling. The results of Morris *et al.* (2000b) showed that stages of *T. bryosalmonae* are equally distributed between anterior and posterior kidney. The remaining kidney and a part of liver and spleen were fixed in formalin for light microscopy. One day after the last sampling, three fish of each group were killed, dissected and kidney, liver and spleen tissues were tested for presence of bacteria.

### 4.2.2 DNA extraction and PCR

Tissue samples were homogenised using a Tissue Lyser® (QIAGEN). DNA was extracted with a QIAamp® DNA Mini Kit (QIAGEN) according to manufacturer's instructions and its concentration was measured photometrically (Eppendorf Photometer). The DNA-concentrations were between 600 and 1100 ng/µL. To avoid inhibition of PCR-reactions, all samples were diluted 10-fold with double distilled sterile water. PKD-specific primers for qRT-PCR were designed (Table 4.2) according to the 18S rDNA sequence of *Tetracapsuloides bryosalmonae* and 18S rDNA sequences of several kidney specific myxozoans from the GenBank™[3] database (GenBank™ accession no. and references are shown in Table 4.1).

**Table 4.1 Sequences used in the present study**

| *Species* | *GenBank accession no.* | *Reference* |
|---|---|---|
| *T. bryosalmonae* | U70623 | Saulnier *et al.* 1999 |
| *Chloromyxum truttae* | AJ581916.2 | Holzer *et al.* 2004 |
| *Myxidium truttae* | AJ582061.2 | Holzer *et al.* 2004 |
| *Sphaerospora truttae* | AJ581915.1 | Holzer *et al.* 2004 |
| *Hoferellus gilsoni* | AJ582062.1 | Holzer *et al.* 2004 |
| *Myxobilatus gasterostei* | AY495703.1 | unpublished |

[3]National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/Genbank/

Additionally, a BLAST[4] search was conducted to detect possible homologies with DNA sequences in the database. To allow quantification of the parasite relative to the amount of host tissue, the insulin growth factor I (IGF I) gene was used as a reference gene to correct the measurements for the amount of trout tissue. For amplification of rainbow and brown trout IGF I, the primers tgIGF1-231 F and tgIGF1-297 R (Kelley *et al.* 2004) were used (Table 4.2). All primers were synthesised by Eurofins MWG Operon.

**Table 4.2 Primers used for qRT-PCR**

| *Primer* | *Sequence* | *Product size/ Reference* |
|---|---|---|
| PKD-real F | 5'- TGT CGA TTG GAC ACT GCA TG -3' | Product: 166 bp/ |
| PKD-real R | 5'- ACG TCC GCA AAC TTA CAG CT -3' | this study |
| tgIGF1-231 F | 5'- CAG TTC ACG GCG GTC ACA T -3' | Product: 67 bp/ |
| tgIGF1-297 R | 5'- CCG TAG CTC GCA ACT CTG G -3' | Kelley *et al.* 2004 |

The specificity of the primer pair was assessed with DNA from a variety of Myxozoa (*Henneguya* sp.*, Myxobolus cerebralis, Mxyidium lieberkuehni, Sphaerospora renicola, Zschokkella* sp. and *T. bryosalmonae*) and DNA from non-infected rainbow trout kidney. To test the PCR-efficiency of both primer pairs, dilution series of DNA from a PKD-infected brown trout kidney were prepared. Reactions for qRT-PCR were set up with iQ Sybr Green Supermix in iCycler iQ 96-well PCR-plates (BIO-RAD Lab-oratories). One reaction contained 12.5 µL 2x Supermix, 0.4 µM of each primer and PCR-grade water to yield 25 µL reaction volume. All samples were run in duplicates with DNA of non-infected fish as negative and DNA of PKD infected fish as positive controls on every plate. Reactions with the PKD-real and IGF-I primer-pair for the respective sample were run together on the same

**Table 4.3 Program for qRT-PCR**

| *Temperature* | *Time* | *Repeats* |
|---|---|---|
| 95 °C | 5 min | *1x* |
| 95 °C | 45 sec | *38x* |
| 61 °C | 45 sec | |
| 72 °C | 45 sec | |
| 95 °C | 60 sec | *1x* |
| 70 °C + 0.5 °C /cycle | 10 sec | *40x* |
| 15 °C | Hold | Infinite |

[4]National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/tools/primer-blast

plate. A melting-curve was created subsequently to the qRT-PCR amplification to detect irregularities or unspecific amplifications. The PCR program used is shown in Table 4.3.

The efficiency (*E*) of the amplification was determined (1) from the slope (*s*) of the DNA-standard curves (Fig. 4.1) and the relative parasite load was calculated according to the formula of Pfaffl (2001) (2). *E* is the amplification efficiency of the primers and *s* the slope of the dilution series regression line; *a* is the ct value of the sample with the lowest parasite load and *n* are all other ct values of the dataset. With this calculation method, the lowest relative expression level will be 1.

$$E = 10^{\frac{-1}{s}} \qquad (1)$$

$$R = \frac{E_{PKD}^{\,ct_{PKDa} - ct_{PKDn}}}{E_{IGF}^{\,ct_{IGFa} - ct_{IGFn}}} \qquad (2)$$

One PKD-real F/R-PCR product of each strain was sequenced to confirm the specificity of the PKD primers. QRT-PCR assays were conducted on a myiQ iCycler with Sybr Green detection and the MyiQ system software v1.0.410 (BIO-RAD Laboratories).

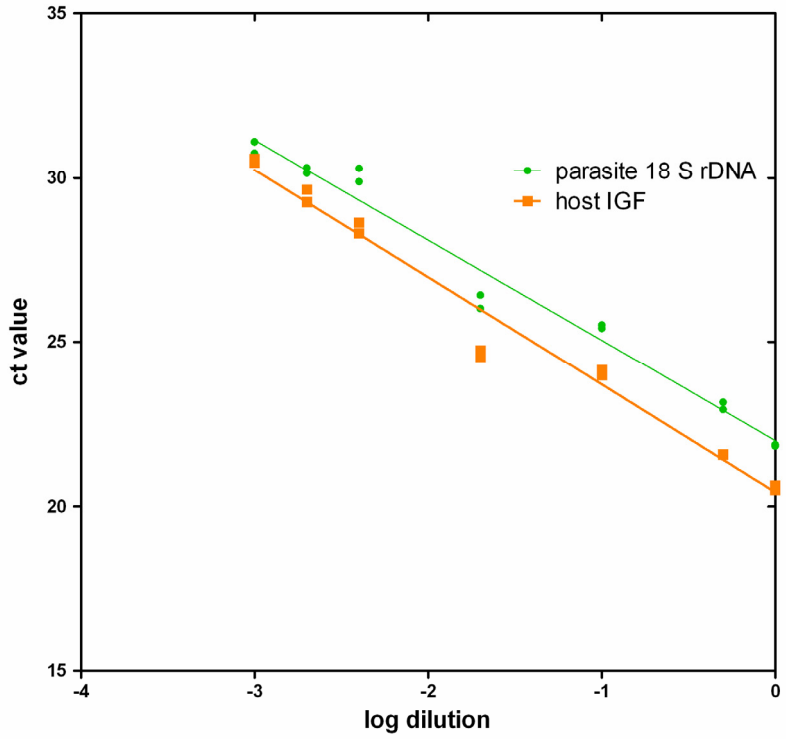


**Figure 4.1:** DNA-standard curves obtained from dilutions series of DNA from a PKD-positive rainbow trout kidney amplified with the PKD-real (green line) and IGF primer pair (orange line).

### 4.2.3 Histology and immunohistochemistry

Tissue samples fixed in 10% neutral buffered formalin (v/v) were washed, dehydrated and embedded in paraffin wax (Tissue-Tek VIP, Sakura Bayer Diagnostics). Sections were cut at 4 µm (1140 Autocut, Reichert-Jung) and processed for immunohistochemistry or stained with haematoxylin and eosin (H&E). For immunohistochemical analysis, one section of each target organ of each fish was stained immunohistochemically with the monoclonal antibody P01 (Aquatic Diagnostics LTD) according to the protocol given in section 2.2.6. A kidney sample of a SPF rainbow trout and a PKD-infected rainbow trout were used as negative and positive controls respectively. Sections were examined for parasite stages and histopathological changes in the tissue with 400-fold magnification.

### 4.2.4 Statistical analysis

Measurements of the mean body-size of each strain/species were analysed by the non parametric Kruskal-Wallis test, as not all groups were normally distributed (D'Agostino and Pearson omnibus normality test) and the variances differed significantly according to Bartlett's test for equal variances. A Dunn's multiple comparison test was performed to detect significant differences between size and weight of the strains.

The qRT-PCR data was $\log_{10}$ transformed to achieve normal distribution and analysed for groups and sampling points by analysis of variance (ANOVA) followed by Bonferroni post hoc test for multiple comparisons of selected groups. Comparison was conducted for all against all means within the groups of each sampling time point (i.e. TL1 vs. BT1, TL1 vs. WT1, etc.). To compare the increase of parasite load over time, regression analyses were conducted to compare the lines fitted to the log-transformed qRT-PCR data per strain/species over all three time points. The correlation between fish size and parasite load was evaluated for each time point using correlation analysis based on the Pearson correlation coefficient. Statistical analyses were conducted with GraphPad Prism v5.00 software.

## 4.3 Results

### 4.3.1 Fish infection

No mortalities occurred before and during the field exposure. Till the end of the experiment 9 BT and 5 WT died, most of them between 2 and 3 wpe. No more ectoparasites could be detected after NaCl-treatment. Inner organs of the 3 fish tested from each group after the experiment were free of bacterial infections. None of the fish showed any external or internal symptoms of PKD (e.g. kidney swelling).

The fish of each group did not grow significantly from 2 to 4 wpe (Kruskal-Wallis test, $p = 0.74$, data not shown). Due to their different age, the fish size varied between the different experimental groups. As this might have consequences for the development of *Tetracapsuloides bryosalmonae*, the size differences were also evaluated statistically. In figure 4.2 the body-length and body-weight measurements of each rainbow trout strain and of brown trout are shown. According to Dunn's test, mean body length and weight of BT, WT and TL differed significantly from both SB and DG. Especially the fish of the DG strain were much bigger than those of all other strains (e.g. from about 1.5 to 2 times longer and 3.7 to 8.3 times heavier than SB and BT respectively).

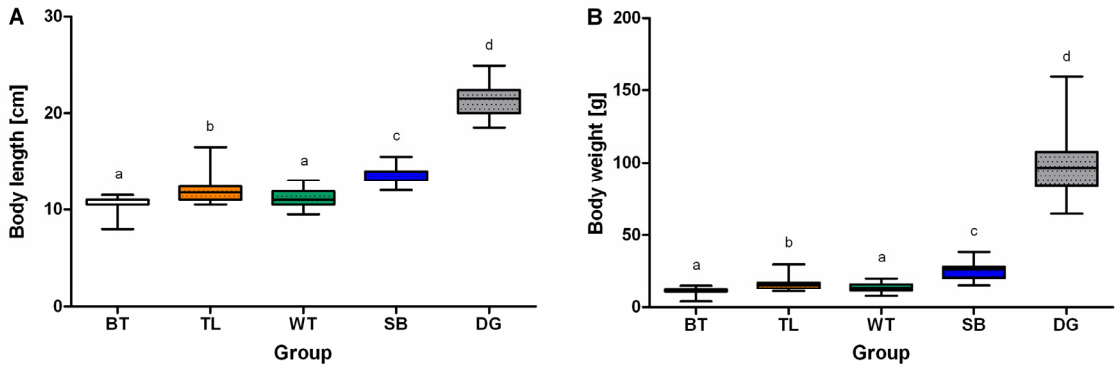


**Figure 4.2:** Box-Whiskers-plot of body length (A) and body weight (B) of all test groups. Significant differences ($p < 0.05$, Dunn's multiple comparison test) are indicated by letters: a vs. c+d, b vs. c+d.

### 4.3.2 Quantitative real-time PCR

The specificity tests with the PKDreal F/R primer pair showed no amplification for trout kidney DNA and DNA of the myxozoan species tested, except DNA of *Tetracapsuloides bryosalmonae*. Also, no unintended matches were found by BLAST-search with the PKDreal-

primers. Sequenced PCR products of each strain were 100% similar to sequences of *T. bryosalmonae* in the GenBank™ (accession no. U70623, Saulnier *et al.*, 1999). The PCR-efficiencies were 2.12 and 2.02 for PKDreal and IGF-I primer pairs respectively. All samples were tested positive for *T. bryosalmonae* in qRT-PCR, resulting in an infection prevalence of 100% for all strains.

Evaluation of the qRT-PCR-quantified relative parasite load revealed the highest parasite intensity in BT at all time points (Fig. 4.3), though no significant difference was detected between BT-, TL- and WT-fish at 2 and 3 wpe. At 4 wpe, the value measured for BT was significantly higher ($p < 0.001$) compared to all rainbow trout strains except TL. SB consistently showed the lowest parasite load, with significant differences to BT at all time points ($p < 0.001$) and compared to the other groups at 2 and 3 wpe (vs. TL 2 and WT 2: $p < 0.001$; vs. TL 1 and WT 1: $p < 0.01$) with the exception of DG ($p > 0.05$) (Fig. 4.3). Infection intensity for DG was low at the first two time points, but besides BT, significant differences could be noted only in comparison to TL 2 and 3 wpe (both $p < 0.05$). At 4 wpe all rainbow trout strains reached an equal level of parasite load, and no significant differences could be detected within TL, WT, SB and DG (Fig. 4.3).

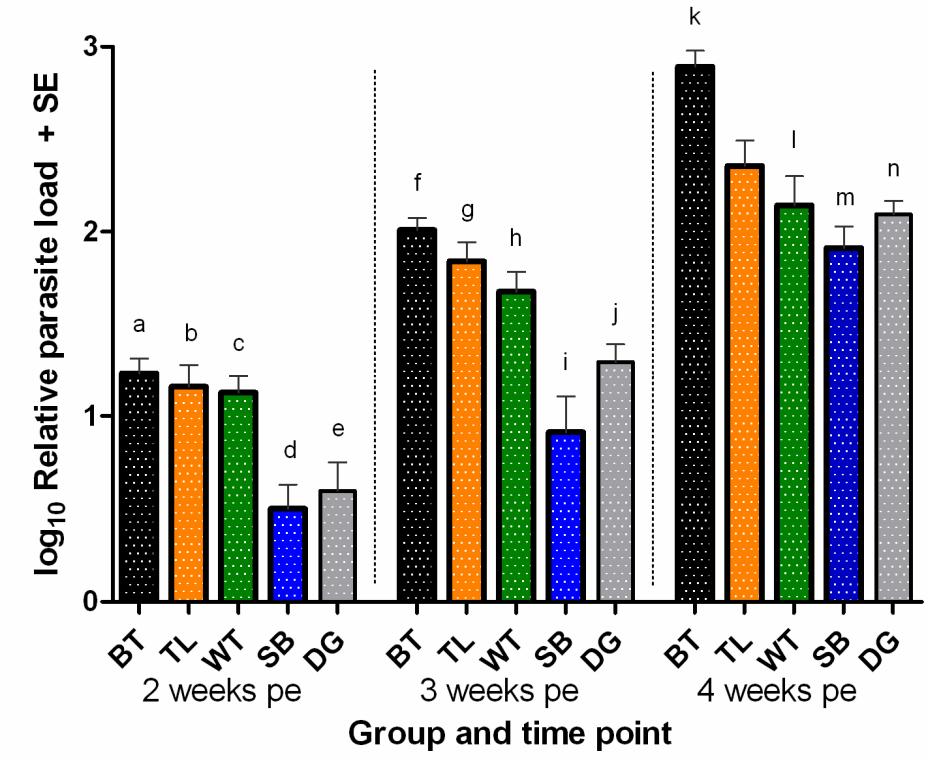


**Figure 4.3:** Mean (+ SE) relative parasite load determined by q-RT-PCR for all test groups at each time point. Significant differences ($p < 0.05$) for comparisons of groups of each time point (Bonferroni test) are indicated by letters: 2: a vs. d+e, b vs. d+e, c vs. d; 3: f vs. i+j, g vs. i+j, h vs. i; 4: k vs. l+m+n.

Change of parasite load over time is illustrated in figure 4.4. As the log-transformed dataset was used to create the graph, the exponential multiplication of the parasite can be approximated by a linear model for the three time points analysed. The increase rate of parasite number, reflected by the slope (s) of the respective line, was highest for BT (s = 0.83). Multiplication speed of the parasite was similar for BT, SB (s = 0.71) and DG (s = 0.75), as the slopes were not significantly different (BT vs. SB: p = 0.32; BT vs. DG: p = 0.40; SB vs. DG: p = 0.75). Also, no significant difference was found comparing the increase rates of parasites in the kidneys of TL (s = 0.60) and WT (s = 0.51; p = 0.45), but the parasite increase rate of the latter two groups differed significantly from BT (TL: p = 0.027; WT: p = 0.0027). Only WT differed significantly from DG with regard to the speed of parasite multiplication (p = 0.042).

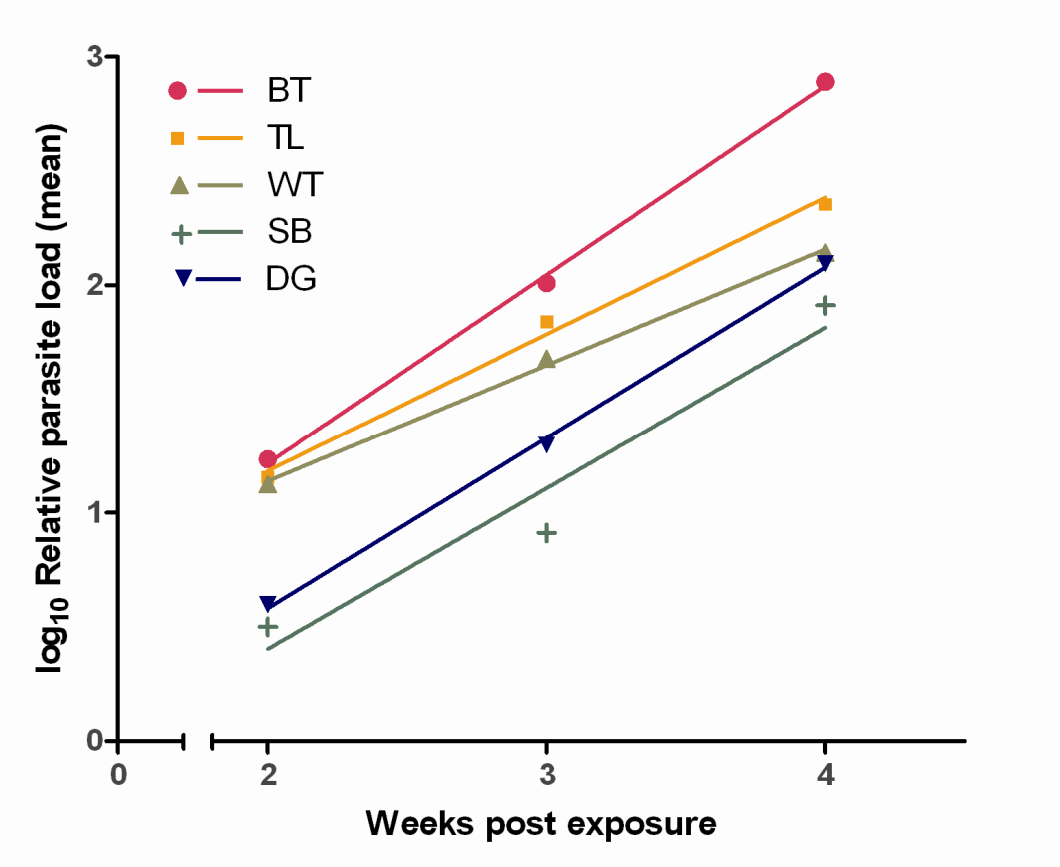


**Figure 4.4:** Linear regression of parasite multiplication rate from 2 to 4 wpe in all test groups. Lines are calculated from values of individual fish measured by qPCR.

The increase rates of parasite numbers from one time point to the next (calculated from non-transformed data) were not constant between the first and the last two weeks of the experiment (Fig 4.5). Especially in BT, SB and DG the parasite multiplied considerably faster between 3 and 4 wpe than between 2 and 3 wpe. Multiplication rate in SB was about 2-fold

higher from 3 to 4 wpe compared to 2 to 3 wpe. In contrast, the multiplication rate of the parasite in WT and TL showed a slight decline over time.

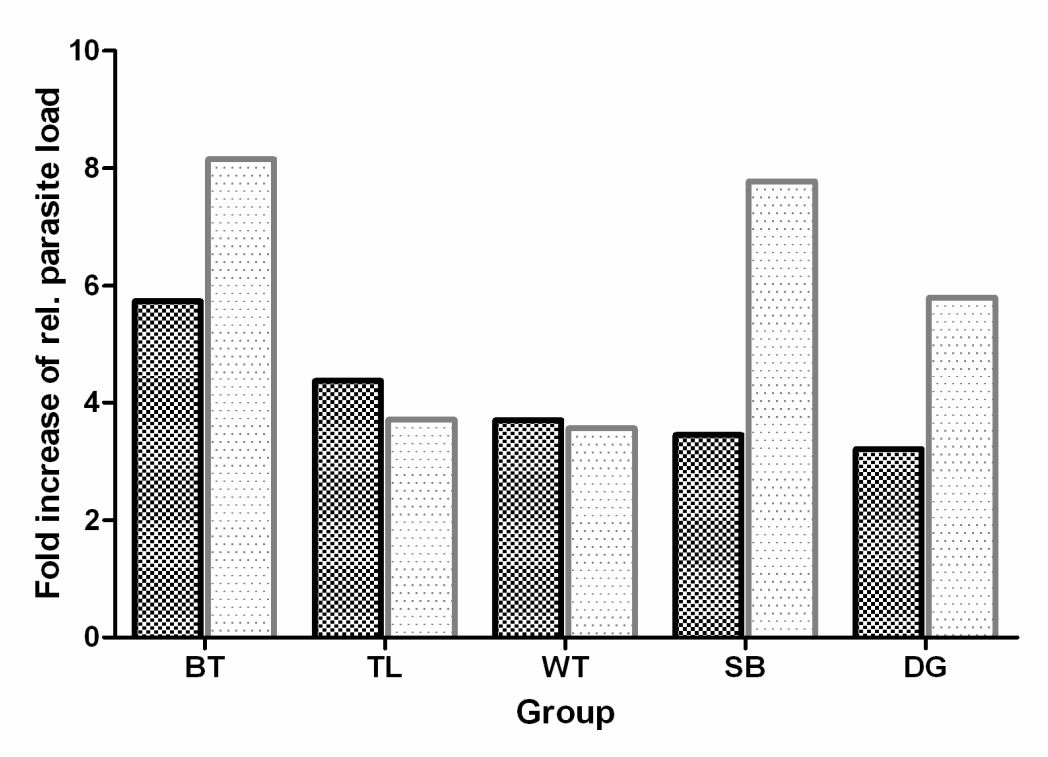


**Figure 4.5:** Increase rate of parasite load in the test groups from 2 to 3 wpe (dark column) and 3 to 4 wpe (light column). Values are calculated from group means of untransformed values of relative parasite load obtained by qPCR.

The correlation analysis of parasite load vs. fish body length for each time point showed a weak negative correlation between the two parameters (Table 4.4, Fig. 4.6). No correlation was observed when the fish of the different strains at each time point were analysed separately (data not shown).

**Table 4.4 Pearson correlation coefficients (r) with 95% confidence interval**

| WPE | r | 95% confidence interval | n | p |
|---|---|---|---|---|
| 2 | -0.47 | [-0.68; -0.19] | 40 | $p < 0.01$ |
| 3 | -0.35 | [-0.59; -0.038] | 40 | $p < 0.05$ |
| 4 | -0.38 | [-0.61; -0.072] | 40 | $p < 0.05$ |

wpe: weeks post exposure; r: Pearson correlation coefficient; n: number of samples; p: level of significance.

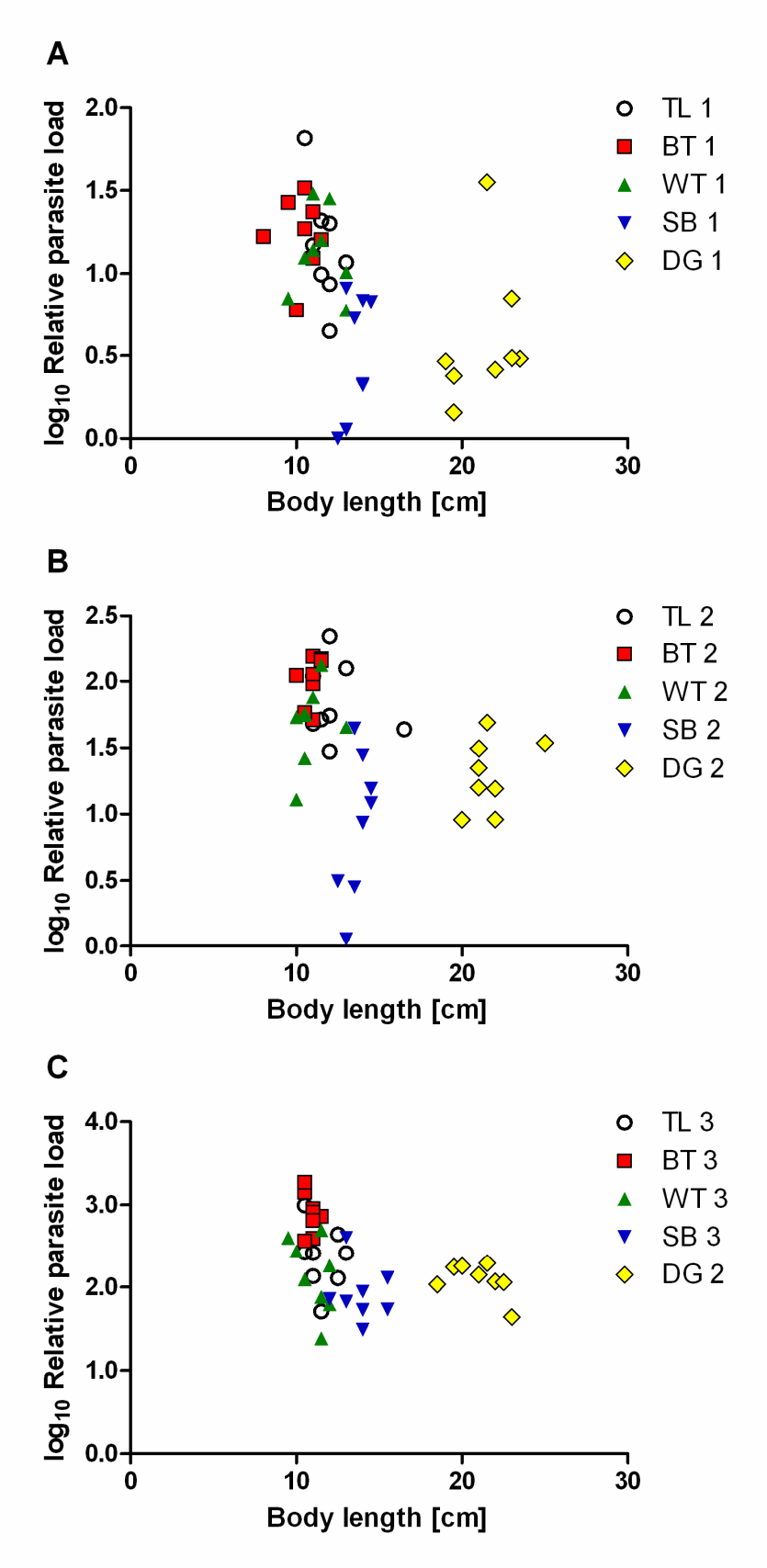


**Figure 4.6:** Correlation analysis of fish body length vs. parasite load determined by qPCR for 2 wpe (A), 3 wpe (B) and 4 wpe (C). Each graph shows data of all fish at one time point.

### 4.3.3 Histology and immunohistochemistry

Surprisingly, no parasite stages were detected in histological kidney-sections of most fish. Using immunohistochemical staining, only a single parasite stage was found in the spleen of one SB fish and one interstitial kidney stage was detected in BT and WT fish. As the number of parasites was very low, no pathological changes could be found in the tissues examined histologically.

## 4.4 Discussion

In the present study, the differential development of *Tetracapsuloides bryosalmonae* in various rainbow trout strains and brown trout was investigated at an early phase of the disease. Resistant strains of fish hosts have been identified for a variety of myxozoan parasites (see Sitjà-Bobadilla 2008 for review). For PKD, differences in susceptibility have previously been observed at the species and strain level. Ferguson & Needham (1978) reported that PKD caused up to 75% mortalities in rainbow trout fingerlings, while Atlantic salmon (*Salmo salar*) showed no such losses, even though both species were held under the same conditions. Brown *et al.* (1991) also observed landlocked Atlantic salmon (*S. salar ouananiche*) to be more resistant to PKD than Arctic char (*Salvelinus alpinus*). Among three species of Pacific salmon, coho salmon (*Oncorhynchus kisutch*) were found to be more resistant than chinook (*O. tshawytscha*) and kokanee salmon (*O. nerka*) (Arkush & Hedrick 1990). A possible strain difference between stocks of Scottish and Norwegian Atlantic salmon (*S. salar*) parr regarding the susceptibility to PKD was suggested by independent observations from two Scottish fish farms (Ellis *et al.* 1985). In both locations, the Scottish salmon were less susceptible to the disease than the Norwegian fish, though both were kept in water from the same supply. A similar case from Scotland was reported by Quigley & McArdle (1998). In the first experimental work com-paring the susceptibility of two rainbow trout strains to *T. bryosalmonae*, no differences in mortality or number of parasite-stages detected in histological sections were recorded (El-Matbouli *et al.* 2009).

In the present study, quantitative data on the early development of *T. bryosalmonae* was obtained by qRT-PCR, which made it possible to objectively compare the susceptibility of 4 rainbow trout strains and brown trout to this myxozoan parasite. QRT-PCR had already been

used successfully for detection and quantification of various pathogens (Monis *et al.* 2005, Gasser 2006) including Myxozoa (Cavender *et al.* 2004, Kelley *et al.* 2004) and also *T. bryosalmonae* (Bettge *et al.* 2009a + b). Using this method, detection and quantification of the parasite became possible, even if parasite densities in the host tissues were too low to be detected by conventional methods, like in the present study. The small number of parasite stages detected in the histological sections and the lack of typical symptoms of PKD can be explained by the early sampling time points 2 to 4 wpe to water containing spores of *T. bryosalmonae*. Also, the number of spores present in the water might have been low, slowing down the onset of the disease. In a previous study, first parasite stages could be localized in the gills of fish exposed to the parasite after 3 d and first interstitial stages of *T. bryosalmonae* in the kidney were detected by histology 1 to 4 wpe according to Morris *et al.* (1997, 2000b), after 3 wpe according to Kent & Hedrick (1986) and according to data of Clifton-Hadley *et al.* (1987) not before 5 wpe. Normally, first symptoms of the disease such as swelling of the kidney appear after about 8 weeks and numerous parasite stages become detectable in histological sections facilitating diagnosis of the disease (Clifton-Hadley *et al.*, 1987). This shows that a small number of parasites can be overlooked during early stages of infection, when conventional histology or even elaborate methods like immunohistochemistry are used, emphasising the effectiveness of molecular methods like qRT-PCR for diagnosis of PKD.

According to the qRT-PCR-data obtained in this experiment, brown trout showed the highest number of parasites at all time points compared to the rainbow trout strains used. The finding that brown trout were more susceptible to PKD than rainbow trout in this experiment is contradictory to results of previous studies. Although no direct comparison with the present study is possible, as different fish strains were used, Clifton-Hadley & Feist (1989) observed that brown trout developed less severe symptoms than rainbow trout. Since those observations were based on fish with clinical PKD, it might be that the further clinical course of the disease is less severe in brown trout than in rainbow trout, as there is evidence that the European strain of the parasite is adapted to the indigenous brown trout (see discussion in section 2.4 and in Morris & Adams 2006). In a study conducted in Scotland, Ellis *et al.* (1985) observed a stronger immune response in brown trout compared to rainbow trout, both with clinical signs of PKD. Possibly, this immune response takes effect later in the progression of the disease and will inhibit an overshooting development of the parasite in brown trout. An effective humoral immune response against *T. bryosalmonae* occurs late in the course of the disease as high numbers of antibody producing plasma cells could be measured in fish not before 7 wpe

to spores of *T. bryosalmonae* (Olesen & Vestergård Jørgensen 1986, MacConnell *et al.* 1989) and an immune response intense enough to mediate resistance against reinfection was found only in fish surviving a clinical disease (Hedrick *et al.* 1985, Clifton-Hadley *et al.* 1986b, Foott & Hedrick 1987). Some surface proteins of *T. bryosalmonae* are highly antigenic and a severe cellular response could be detected in *T. bryosalmonae*-infected rainbow trout, involving the proliferation of lymphocytes, causing the symptoms of the disease (Chilmonczyk *et al.* 2002). Therefore, it was assumed, that both cellular and humoral components participate in the reaction against the parasite, with the cellular reaction being more important (Hedrick *et al.* 1985, Klontz *et al.* 1986, MacConnell *et al.* 1989). Obviously, the parasite has some defence strategies against the immune cells, because extrasporogonic stages are regularly enclosed by host phagocytes without being destroyed (MacConnell *et al.* 1989, Kent & Hedrick 1986, Morris *et al.* 2000c, Morris & Adams 2008). It was suggested that the electron dense bodies found closely associated with the parasites plasma membrane play a role in the defence against host immune cells (Angelidis *et al.* 1987, MacConnell *et al.* 1989). As the fish in the present study were sampled before an effective humoral immune response could eliminate parasite cells, the differences observed are probably caused by differences in the response of the innate immune system.

Even though the speed of parasite multiplication for BT, SB and DG was similar, the number of parasites in an equal volume of kidney tissue was higher in BT, which might be explained by the larger size of SB and DG fish. As *T. bryosalmonae* most likely disseminates in the body of the host via the blood stream (Kent & Hedrick 1986, Morris *et al.* 2000b, Holzer *et al.* 2006), the proliferation will already start with a higher density of parasite stages in kidneys of smaller fish compared to larger fish, assuming equal numbers of sporoplasm cells having entered the body. Attachment and penetration of higher spore numbers to larger SB and DG fish due to their larger skin or gill-surface is possible, but did not lead to a similar density of parasite DNA like in the kidneys of BT. Other explanations for differences in the initial relative parasite load might be the mechanical barrier of the host, the early phase response, against penetrating sporoplasms or parasite stages in the blood, which are likely to be influenced by fish age. In that case, it was possible for the parasite to penetrate the younger BT (6 months), and also the TL and WT (both 8 months) in higher numbers than the older SB (9 months) and DG (16 months). Further development of *T. bryosalmonae* seemed to be independent of the age of the fish, as speed of multiplication was similar in BT, SB, and DG. Proliferation of *T. bryosalmonae* in TL and WT was relatively constant and slow compared to

the other groups. Therefore, parasite load of TL and WT is approaching that of SB and DG at 4 wpe. This indicates a possible higher resistance of the TL and WT strains against PKD, as these fish seem to be able to slow down parasite development compared to all other groups. It can be concluded that the seemingly higher resistance of the larger SB and DG fish is only due to their size or resistance mechanisms that prevent stages to enter the fish and to reach the kidney (see above). This effect will most likely be compensated by faster increase of parasite stages in the further course of infection.

The negative correlation of fish size and relative parasite load reflects the lower relative parasite load of the strains SB and DG especially at 2 wpe. At 3 and 4 wpe the correlation is only weakly supported taking into account the wide confidence intervals. The analysis of each strain separately at each time point revealed no relationship between size and infection intensity within one strain, though eight fish are not enough to draw definite conclusions about size-dependency of relative parasite load.

*Myxobolus cerebralis*, the causative agent of Whirling Disease in salmonid fish, is the best studied example concerning resistance of different trout strains to a myxozoan parasite. In previous studies, a German rainbow trout strain (HO) was identified to be more resistant to the disease than the American TL strain (Hedrick *et al.* 2003), which was also used in the present study. This finding was explained by longer time of adaptation and also intended selection of the German strain for increased resistance to *M. cerebralis*. This could also be the reason for the resistance of the WT strain. These fish were raised from a wild population of rainbow trout in Germany, which probably was in contact to PKD and therefore adapted to some degree to *T. bryosalmonae*. Results of a recent study on pathogen resistance of Atlantic salmon populations indicate that myxozoan infections can lead to the decrease of MHC alleles promoting susceptibility to the parasites and therefore select for increased resistance in the host population (Dionne *et al.* 2009). To the authors' knowledge, none of the other trout strains used in the present study were constantly adapted to PKD or selected for resistance to this disease. The higher resistance of the TL strain must be a rather incidental pre-adaptation to infections with *T. bryosalmonae*. It is notable, that the *M. cerebralis*-susceptible TL strain was found to be resistant to *T. bryosalmonae* in the present study. Additionally, in a previous work it was shown that the susceptibility to PKD of the above mentioned Whirling Disease-resistant HO strain was similar compared to TL (El-Matbouli *et al.* 2009). A similar situation was observed for a rainbow trout strain being resistant to the myxozoan *Ceratomyxa shasta*, which was on the other hand rather susceptible to *M. cerebralis* (Hedrick *et al.* 2001). These

findings indicate that resistance of a certain fish strain to one myxozoan parasite does not allow conclusions about resistance of the same strain to other myxozoan species.

Age and size differences of the trout strains investigated in the present study make the interpretation of the results difficult, but nevertheless the data indicates that the brown trout strain investigated was more susceptible to *T. bryosalmonae* than the rainbow trout strains. Additionally, two rainbow trout strains could be identified being able to slow down the development of *T. bryosalmonae* in the kidney of infected fish compared to the other strains of rainbow trout and brown trout. The use of resistant fish for trout farming in areas enzootic for PKD could be a way to reduce disease-related losses. Further studies over a longer period of time will be required to investigate the impact of PKD on susceptible and resistant trout strains at later stages of the disease. Breeding experiments would be necessary to clarify if there is really a genetic basis for the observations from the present study. Also, mechanistic studies are required to find out if these susceptibility differences are caused by the innate immune system or specific immune responses.

# 5. INVESTIGATIONS ON THE LIFE-CYCLE OF *BUDDENBROCKIA*

## 5.1 Introduction

In contrast to the numerous Myxosporea, only three malacosporean species have been described to date. They are found as worm or sac-like parasites in freshwater bryozoans. One of them is *Tetracapsuloides bryosalmonae*, causing proliferative kidney disease (PKD) in salmonid fish and the other two are *Buddenbrockia plumatellae* (Schröder 1910), found in various species of freshwater bryozoans and the recently described *B. allmani*, which seems to be host specific to the rare bryozoan *Lophopus crystallinus* (Tops *et al.* 2005, Hill & Okamura 2007, Canning *et al.* 2007). The existence of further *Buddenbrockia* species can be assumed according to 18S rDNA and protein encoding sequence data (Tops *et al.* 2005, Jiménez-Guri *et al.* 2007b), differing considerably from the sequences of the three species known to date. Differences in spore size and morphology found in two worm-shaped *Buddenbrockia* species from the bryozoan *Plumatella repens* (Morris *et al.* 2002a, McGurk *et al.* 2006b) and a sac-shaped *Buddenbrockia* sp. parasitizing *Cristatella mucedo* (Canning *et al.* 1996) substantiate this assumption.

Up to now, among the Malacosporea the vertebrate host is known only for *T. bryosalmonae* (Anderson *et al.* 1999b, Feist *et al.* 2001, Morris & Adams 2006a), while the propagation of other malacosporeans remains unclear. Several evidences point out the possible existence of further fish-infecting malacosporeans. Reports about bryozoan colonies (*Plumatella magnifica*), infected with malacosporeans in lakes where neither pike (*Esox lucius*) nor salmonids as potential host were present (Anderson *et al.* 1999b, Okamura *et al.* 2001) and the observation of only one rare cyprinid species in an area where infected bryozoans were found (Okamura & Wood 2002) indicate that some malacosporeans possess undescribed fish hosts. This idea is further substantiated by developmental stages with characteristics of malacosporeans found in pillar cells and endotheliocytes of gills and in cells of the blood vessel endothelium of brain and kidney of common carp (*Cyprinus carpio*) (Voronin 1993, Voronin & Chernysheva 1993). On the other hand, Hill & Okamura (2007) found colonies of the

bryozoan *L. crystallinus* raised in the laboratory from dormant sages (statoblasts) to be positive in polymerase chain reaction (PCR) for malacosporean DNA, indicating possible vertical transfer of these parasites via statoblasts. It can be assumed that different life-cycle strategies exist within the class Malacosporea and not all malacosporeans may require a vertebrate host. Aim of this study was to find possible fish hosts for representatives of the genus *Buddenbrockia*. To this end, laboratory infection experiments were conducted. Bryozoans, overtly infected with worm-shaped malacosporean stages, were cohabitated with parasite free fish and vice versa. A molecular phylogenetic analysis including all known *Buddenbrockia* sequences was performed to elucidate the systematic relationships within this group.

## 5.2 Materials and Methods

### 5.2.1 Bryozoa

A variety of bryozoan colonies was collected in October 2008 in a pond (Bronner Weiher, Bavaria, Germany; 48°01'00.00'' N, 10°46'59.65'' E) and kept in the laboratory culture system described in section 2.2.2 at a temperature of 15 °C. Colonies growing side by side on submerged pieces of wood were examined with a dissecting microscope to detect malacosporean infections. By using the key of Wood and Okamura (2005), three bryozoan species were identified as *Plumatella fruticosa*, *P. repens* and *Cristatella mucedo*. In spite of careful examination of the collected substrata, no other bryozoan species were detected. *P. fruticosa* was the least abundant species with 4 small colonies of approximately 2 - 3 cm diameter. Colonies of *C. mucedo* were also small (1 cm length and 0.5 cm width), but about 10 of them were present on the sampled material. *P. repens* showed the largest colonies with approximately 2 - 10 cm diameter. Individual colonies were often fusing and therefore their number can only be estimated to about 5 - 8. Overt infection with wormlike malacosporeans was found only in *P. repens*. One infected zooid containing two worms was sampled and frozen at -20 °C for DNA extraction.

Upfloating statoblasts from the *P. repens* colony containing the malacosporeans were collected and approximately 100 were frozen for subsequent DNA extraction. Approximately 50 statoblasts were germinated to produce new colonies by pipetting them under 2 inverted

Petri-dishes floating in a small box with dechlorinated tap water at 20°C to stimulate hatching. Attempts to germinate statoblasts from *P. fruticosa* and *C. mucedo* failed.

### 5.2.2 Fish

Specific pathogen free (SPF) carp (*Cyprinus carpio*) (6 – 8 cm) were raised from eggs in the laboratory. SPF young of the year brown trout (*Salmo trutta*) (5 – 6 cm) were obtained from a local hatchery and minnow (*Phoxinus phoxinus*) (4 – 5 cm) were purchased from a pet shop. All fish were kept in aquaria with flow through dechlorinated tap water and fed with a commercial diet of fish pellets (BioMar). As the minnow were not certified SPF, liver, kidney and spleen of six specimens were prepared for PCR to test for malacosporean infections. Additionally, carp from two natural ponds (fish farm Wielenbach 47°53'5.00'' N, 11°09' 17.00'' E and fish farm Seeshaupt 47°49'4.00'' N, 11°17'14.00'' E) were obtained to test for the presence of malacosporeans.

### 5.2.3 Fish infection and sampling

The fish (6 carp, 3 minnows and 3 brown trout) were exposed to the above mentioned collection of bryozoan species including the overtly infected *Plumatella repens* in a 50 L aquarium without flow through. After 1 week the carp, minnow and trout were separated from the bryozoans and transferred to separate 20 L aquaria with flow through dechlorinated tap water. During and after the exposure, temperature was adjusted to 18 °C ± 1 °C, and the fish were fed daily.

One fish from each species was sampled at 3, 4 and 5 weeks post exposure (wpe) and the remaining carp were sampled at 8, 10 and 18 wpe. The fish were euthanized with MS222 anaesthetic (Tricaine-methane-sulphonate; Sigma-Aldrich) and kidney, spleen and liver of all fish sampled after 3 wpe were excised and frozen at -20 °C for DNA extraction. At the remaining time points, samples of the kidney were taken for DNA extraction and pieces of all inner organs were fixed in 10% neutral buffered formalin for histology. Small pieces of liver, spleen and kidney were fixed in 2.5% glutaraldehyde in phosphoric buffer according to Soerensen (v/v, pH 7.4) for electron microscopy. Blood smears and squash preparations of the kidney were prepared from all fish and examined for parasite stages.

After 7 wpe of fish to field-collected bryozoans, statoblast-raised *P. repens* were cohabitated for 8 h per day with the exposed carp. For the remaining time the colonies were transferred back to the laboratory culture to allow feeding. These colonies were examined visually every second day for presence of malacosporean stages and samples from the tip of a growing branch, comprising 4 to 5 zooids were taken for PCR at 3, 4, 6 and 8 weeks after the beginning of exposure to the fish. Two carp from each natural pond were dissected and kidney samples were taken for molecular analysis.

### 5.2.4 DNA extraction and PCR

Tissue and statoblast samples were homogenised using a Tissue Lyser® (QIAGEN) and DNA was extracted with a QIAamp® DNA Mini Kit (QIAGEN) following the manufacturer's protocol. For detection of malacosporean parasites, specific primers amplifying a product of about 640 bp of the malacosporean 18S rDNA (mala-f/mala-r; Table 5.1) were designed by comparison of a variety of myxozoan 18s rDNA sequences from the GenBank™[5]. Specificity of the mala-f/mala-r primer pair was tested by a BLAST search[6] for homologies with DNA sequences of other species in the database and by PCR with DNA of uninfected fish tissue, DNA from laboratory raised Bryozoa (*Fredericella sultana*) and DNA of several myxozoans (*Tetracapsuloides bryosalmonae*, *Buddenbrockia plumatellae*, *Myxobolus cerebralis*, *Henneguya* sp.*, Sphaerospora renicola*). For sequencing, a primer-pair (budd f/budd r; Table 5.1)

**Table 5.1 Primer-sequences and PCR-programs**

| *Primer Name* | *Sequence* | *Cycling conditions* |
|---|---|---|
| mala-f | 5' - AAA CGA RTA AGG TCC AGG TC - 3' | 95 °C - 5 min |
| | | 95 °C - 45 sec<br>61 °C - 45 sec<br>72 °C - 45 sec } 40x |
| mala-r | 5' - CAC CAG TGT AKC CCG CGT - 3' | 72 °C - 5 min |
| budd-f | 5' - CTG CGA TGT ACT CGT CTT AAA G - 3' | 95 °C - 5 min |
| | | 95 °C - 45 sec<br>58 °C - 45 sec<br>72 °C - 2:20 min } 40x |
| budd-r | 5' - CGA CCA AGC TCA AAC AAG TTT - 3' | 72 °C - 10 min |

[5]National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/Genbank/
[6]National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/tools/primer-blast/

amplifying a 1784 bp fragment of the malacosporean 18S rDNA was designed. Amplification with both primer pairs was carried out in 20 µL reaction volume containing 10 µL 2x ReddyMix PCR Master Mix (ABGene), 0.5 µmol of each primer and 1 µL DNA. The mixture was topped up to 20 µl with distilled water. All PCR-cycling-conditions are given in Table 5.1. Primers were obtained from Eurofins MWG Operon. If sequencing was desired, the PCR-products were purified with a QIAquick PCR-purification Kit (QIAGEN) and sent for sequencing (GATC Biotech).

### 5.2.5 Phylogenetic analysis

The obtained partial 18S rDNA sequences of malacosporeans were aligned with Clustal W (Thompson *et al.* 1997), together with the sequences of an unknown, worm-like malacosporean from *Fredericella sultana*, of *Buddenbrockia allmani*, of a sac-like and a worm-like *B. plumatellae* and a sequence of *Tetracapsuloides bryosalmonae* (Table 5.2).

**Table 5.2 Sequences used for phylogenetic analysis**

| *Isolate* | *GenBank accession no.* | *Reference* |
|---|---|---|
| worm-like malacosporean from *F. sultana* | AJ937879 | Tops *et al.* 2005 |
| *B. allmani* | AJ937880 | Tops *et al.* 2005 |
| sac-like *B. plumatellae* | AJ937881 | Tops *et al.* 2005 |
| worm-like *B. plumatellae* | AY074914 | Monteiro *et al.* 2002 |
| *T. bryosalmonae* | U70623 | Saulnier *et al.* 1999 |

The resulting multiple alignment was corrected visually for inconsistencies using the BioEdit software v7.0.9 resulting in a final alignment of 1,055 bp. Phylogenetic analysis was conducted with the Bayesian inference software MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). Modelgenerator v0.85 (Keane *et al.* 2006) was used to determine the evolutionary model for this analysis. A general time reversible model (GTR + I + Γ) with rate variation across sites estimated by a gamma-distribution (four rate categories) was found to be most appropriate. Two independent runs were conducted with 4 chains and 1,000,000 generations. Trees were sampled every 100 generations. The first 25 % of the samples were discarded (burn-in-phase) and a 50 % majority-rule consensus tree was created. The resulting tree was

visualized with MEGA v4 (Tamura *et al.* 2007). The multiple alignment used for this analysis is shown in the appendix.

### 5.2.6 Light and electron microscopy

The formalin fixed samples were processed as described in section 2.2.6, immuno-histochemistry was conducted according to section 2.2.6 and processing of electron microscopy samples was described in section 3.2.4. Blood smears were stained with Diff-Quick (Andwin Scientific).

## 5.3 Results

### 5.3.1 Primer specificity

The specificity tests with the mala-f/r primers did not result in amplification of DNA from uninfected fish, bryozoans or the myxozoans tested, except the intended template of the 18S rDNA of *Tetracapsuloides bryosalmonae* and *Buddenbrockia* sp. No unintended matches were found in BLAST-search. The budd f/r primer pair successfully amplified the desired parasite DNA sequence and did not produce any unspecific bands.

### 5.3.2 Bryozoa

Two days after transfer to the laboratory, an overt malacosporean infection was observed in three zooids of *Plumatella repens* in a colony comprising several hundred individual zooids (Fig. 5.1). The morphology of the parasite stages was similar to those described previously for worm-shaped *Buddenbrockia plumatellae* (Canning *et al.* 2002, Morris *et al.* 2002a, McGurk *et al.* 2006b). The worms showed fast bending and coiling movements. After two weeks in the laboratory most of the bryozoans used for fish infection died and no more malacosporean stages were found in the remaining zooids. The DNA extracted from the infected zooid was amplified with the budd f/r primer pair. Blast search[7] with the resulting product (1,674 bp;

[7]National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi

GenBank accession no. FJ939289) revealed highest similarity (99.5 %) to *B. plumatellae* (accession no. AY07-4914; Monteiro *et al.* 2002).

No malacosporean DNA could be detected by PCR with the mala f/r-primers in the statoblasts collected from the infected colony. However, *P. repens* colonies raised from statoblasts were PCR positive after cohabitation with exposed carp for 3 weeks. All further samples taken from the exposed bryozoan colonies at 4, 6 and 8 wpe to the carp gave a negative result with PCR. No overt infection of the exposed bryozoans could be observed within this period.

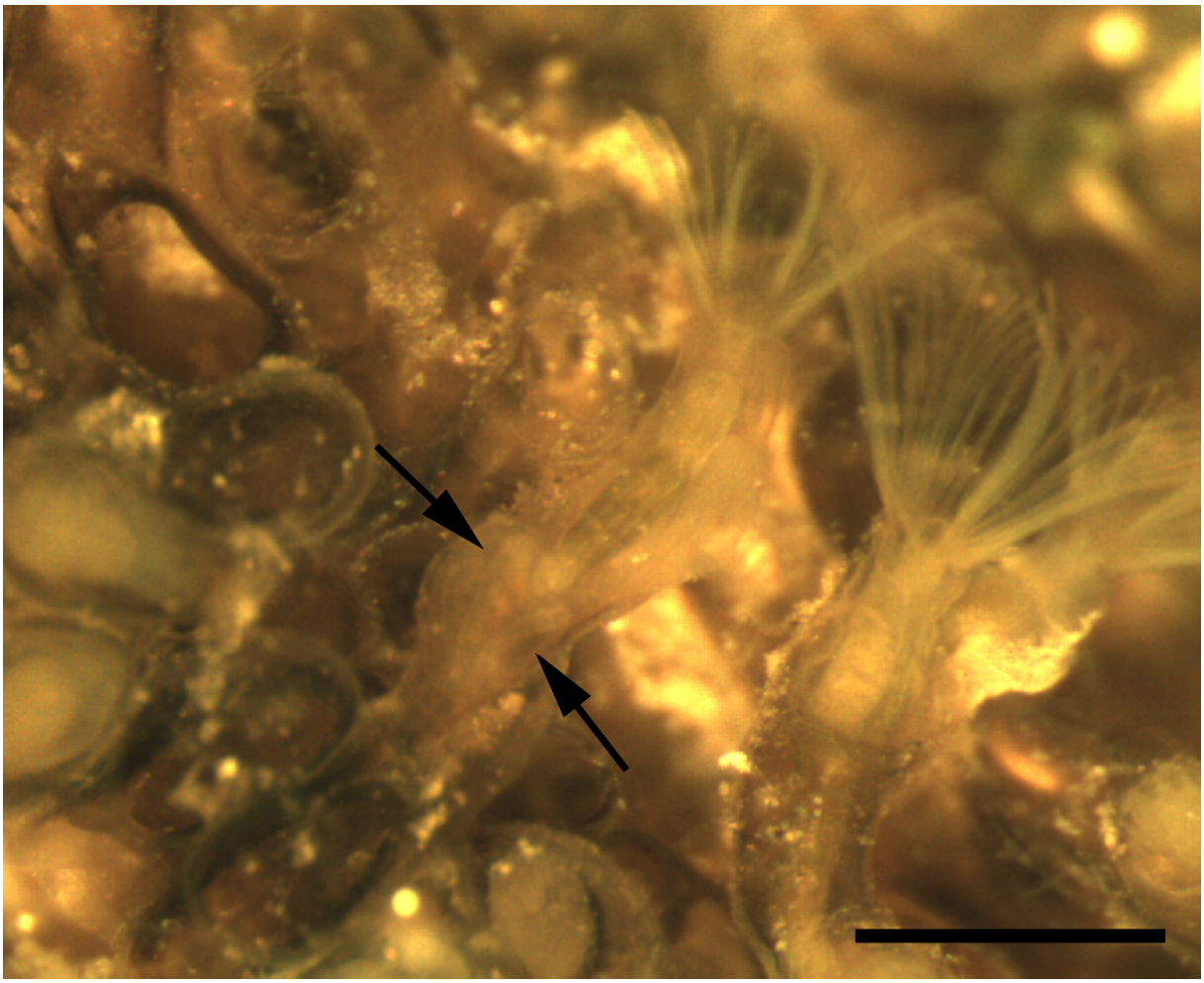

**Figure 5.1:** Zooid of *P. repens* overtly infected with *B. plumatellae*. Arrows point to the position of the "worm". Bar: 500 µm.

### 5.3.3. Fish Infection

The PCR and sequencing results are summarized in Table 5.3. No malacosporean DNA was detected in any of the six minnow-samples tested before the infection experiment. None of the fish cohabitated with infected Bryozoa showed any external symptoms or any abnormities in inner organs during the course of the trials. DNA from liver, spleen and kidney of the carp

sampled after 3 wpe showed the expected band on an agarose gel after PCR-amplification with the mala f/r primers. Among the three organs, the strongest band was detected in the kidney sample. Of the samples taken from brown trout and minnow after 3 wpe, only the kidney samples were PCR positive.

For the carp sampled after 3 wpe, the PCR-product from liver tissue was also sequenced to test if the parasite found there was identical to the one in the kidney. All further kidney samples from carp were PCR positive and all sequences obtained from this fish species were 100 % identical. Only the minnow sampled at 4 wpe was also tested positive by PCR, while the kidney of the minnow sampled one week later was negative. Sequencing showed that the two sequences from minnow were 100 % identical. The remaining brown trout tested negative by PCR. Surprisingly, the sequenced segments of the 18S rDNA of the parasites from carp (1,626 bp; GenBank accession no. FJ939290) and minnow (1,679 bp; accession no. FJ939291) differed considerably (3.7 %). The sequence obtained from kidney of minnow was 99.5 % similar to a *Buddenbrockia plumatellae* 18S rDNA sequence from France (GenBank accession no. AY074914; Monteiro *et al.* 2002), and 100 % similar to the sequence from the Malacosporea-infected *Plumatella repens* collected in the present study. The sequence of the parasite from carp was most similar to the sequence AJ937880 (Tops *et al.* 2005) of *B. allmani* (97.8 %).

The sequence (569 bp) obtained from brown trout (accession no. FJ939294) was 99.6 % similar to a sequence of *Tetracapsuloides bryosalmonae* in GenBank (accession no. U70623; Saulnier *et al.* 1999), indicating that some of the bryozoans collected in the present study must have been infected with *T. bryosalmonae*, which was transmitted to exposed brown trout. Of the 4 carp from the natural ponds, 3 were tested positive for Malacosporea by PCR (pond 1; 989 bp: accession no. FJ939292; pond 2; 1,087 bp: FJ939293). The sequences of these isolates were 100 % similar to those obtained from the laboratory-infected carp.

### 5.3.4 Phylogenetic analysis

The 6 *Buddenbrockia*-sequences used for phylogenetic analysis were very similar. Therefore, the number of phylogenetically informative sites was low and the placement of the different species was not resolved properly (Fig. 5.2). Nevertheless, the genetic separation of sac-like and worm-like *Buddenbrockia plumatellae* was supported by high posterior probability. The worm-like *B. plumatellae* from France (GenBank accession no. AY074914; Monteiro *et al.*

2002) and the worm-like malacosporean from *Plumatella repens* infecting minnows found in the present study were grouped together closely. In this consensus-tree, the carp-parasite clusters with the *B. plumatellae* clade, but with low probability of only 59 %. Also, the placement of the unknown *Buddenbrockia* sp. from *Fredericella sultana* (AJ937879; Tops *et al.* 2005) and *B. allmani* (AJ937880; Tops *et al.* 2005) is not reliable and therefore these branches were collapsed. (Fig. 5.2).

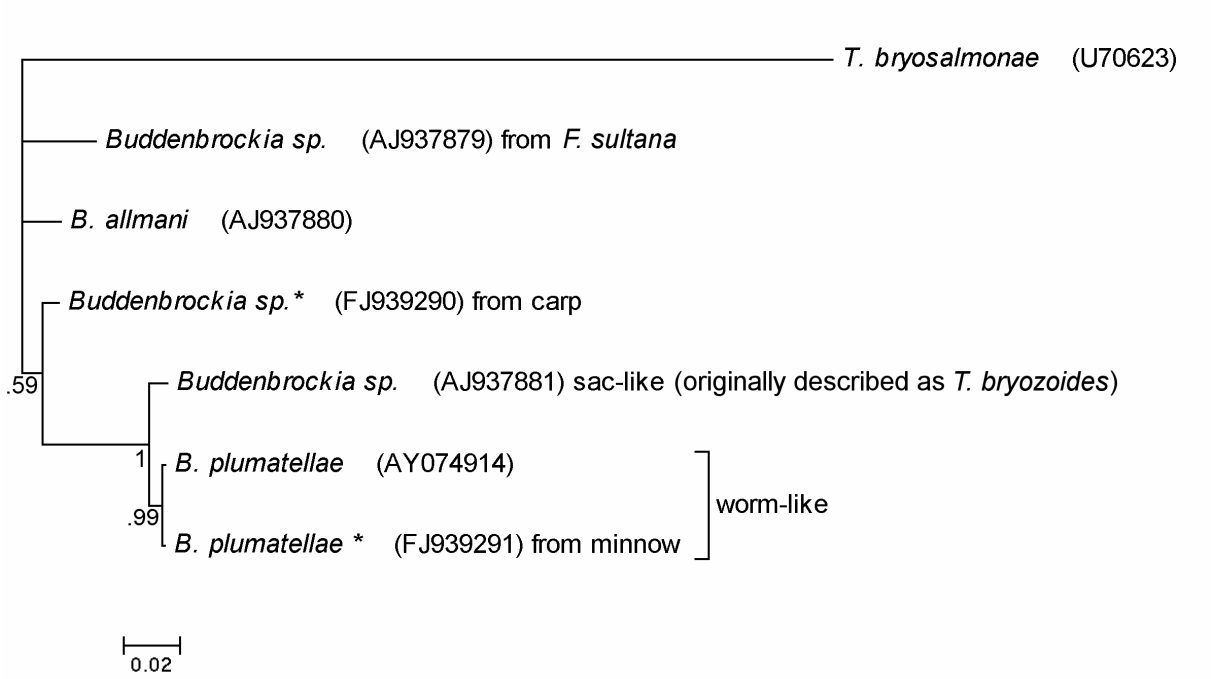


**Figure 5.2:** Molecular phylogeny of the genus *Buddenbrockia*. Results of Bayesian analysis are shown in a 50 % majority-rule consensus tree with *T. bryosalmonae* as an outgroup. Posterior probabilities are given at the nodes. Respective GenBank accession numbers are given behind the species names in parentheses. Asterisks indicate species from the present study. If it was necessary to distinguish the *Buddenbrockia* species, the respective host or an explanation is given behind the genus name.

### 5.3.5 Light and electron microscopy

Malacosporean stages were first observed in H&E stained paraffin and epon semi-thin sections of organ samples from carp at 5 wpe, when several intratubular stages were visible in the kidney-sections of the carp (Fig. 5.3A). At 8 wpe, sporogonic stages were seen in light and electron microscopy of the carp kidney including maturing, elongated spores with 2 polar capsules (Fig. 5.3B). Longitudinal sections of infected kidney tubule segments revealed numerous adjacent groups of parasite cells in different stages of sporogony. A maximum

number of 4 small and 1 larger cell could be found within one primary cell (Fig. 5.3C). Processes were observed that seemed to attach the parasite cell via the tubular epithelium (Fig. 5.3B). In the carp sampled 10 and 18 wpe the infection either was less severe or was already declining, because only a few stages similar to those seen at 8 wpe were found in the kidney tubules.

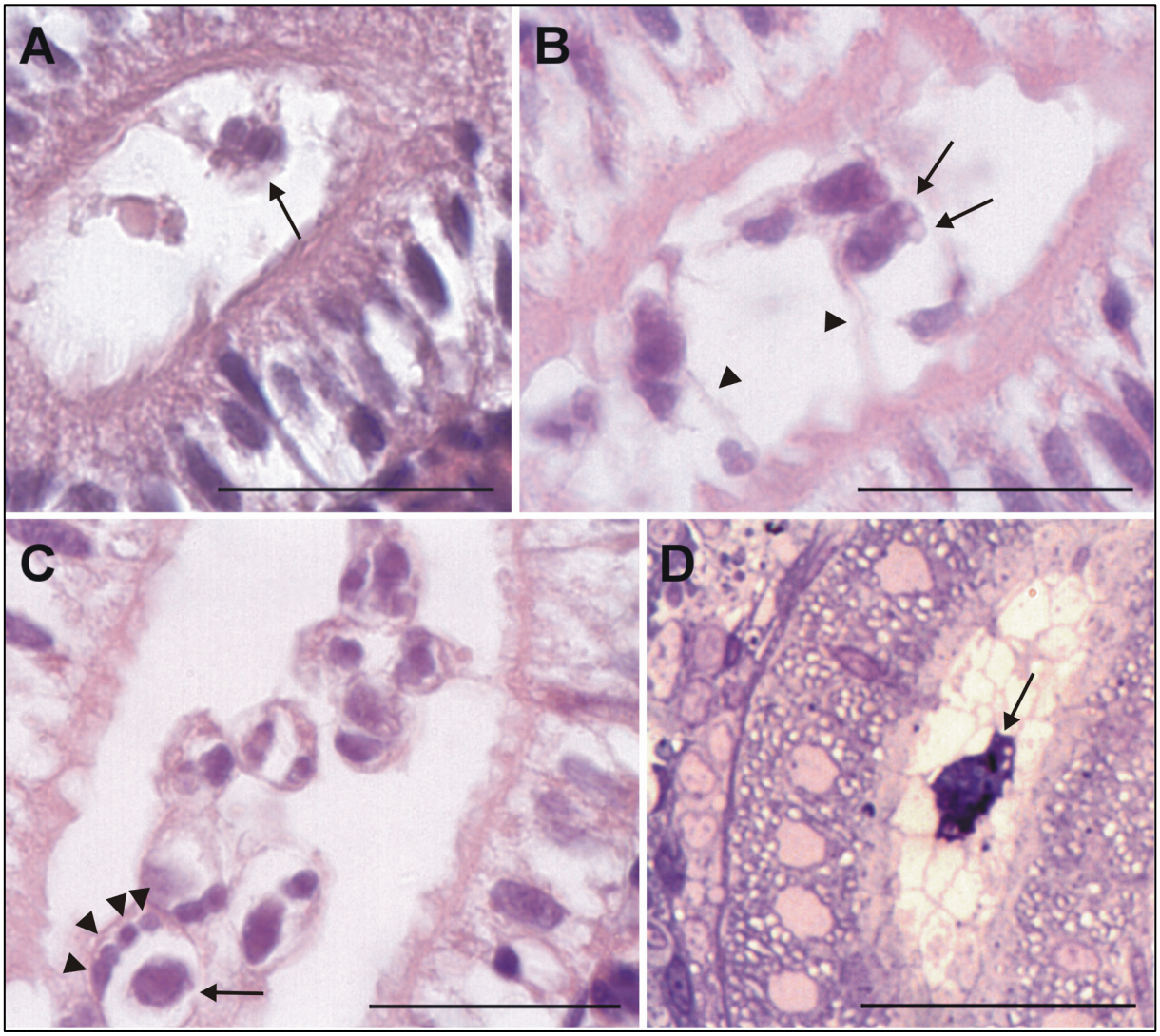


**Figure 5.3:** Light microscopy of parasite stages in kidneys of carp and minnow. **A**. Paraffin section of sporogonic stage (arrow) in kidney tubule of carp sampled at 5 wpe. H&E stain. **B**. Paraffin section of sporogonic stages in kidney tubule of carp sampled 8 wpe. One maturing spore with developed polar capsules is shown (arrows). The parasite cells are connected to the tubular epithelium by cytoplasmic processes (arrowheads). H&E stain. **C**. Paraffin section of sporogonic stages in kidney tubule of carp sampled at 8 wpe. Four small cells (arrowheads) and one larger cell (arrow) can be seen in one of the primary cells. H&E stain. **D**. Semi-thin section of parasite stage in kidney tubule of minnow sampled 4 wpe (arrow). Toluidine blue stain. Bar: 25 µm.

As the minnow sampled 5 wpe tested negative for malacosporeans by PCR, histological examination was conducted only for the samples taken 4 wpe. In kidney samples of this time-point, very few intraluminal, single cell stages were observed only in semi thin sections (Fig. 5.3D). Presporogonic parasite stages outside the kidney tubules or in samples of liver and spleen of both carp and minnow were not observed. No IHC staining of *Buddenbrockia*-stages using the monoclonal antibody specific for *Tetracapsuloides bryosalmonae* could be achieved. Parasite stages were detected neither in squash preparations nor in blood smears.

In electron microscopy, sporogonic stages were found in kidney tubule sections of the carp sampled 8 wpe. Stages of different maturation were seen within kidney tubules consisting of a primary cell enclosing the sporogonic cells thereby forming a pseudoplasmodium. The sporogonic cells comprised one cell doublet (secondary and tertiary cell) and a group of secondary cells without tertiary cells (Fig 5.4A). Analogous to the light microscopic observations, the greatest number of secondary cells seen in electron microscopy was five (Fig. 5.4B). Developing parasite cells seemed to anchor to microvilli of tubular epithelial cells by pseudopodia-like processes (Fig. 5.4C). Two capsulogenic cells forming one polar capsule each developed inside a secondary cell (Fig. 5.4D). In the most mature spore stage found, one sporoplasm bearing sporoplasmosomes and one of the capsulogenic cells could be seen. Valvogenic cells surrounded almost the entire spore (Fig. 5.4E). Maturing polar capsules with polar filament were visible in some sections (Fig 5.4F). In contrast to those from carp, stages from minnow were only detectable up to 4 wpe. At this time, only few, apparently early sporogonic stages, were present in kidney tubules. No secondary cells were observed (Fig. 5.5A). Some of these cells contained numerous vacuoles containing bundles of electron dense material. Cytoplasmic processes of parasite cells seemed to enclose host material (Fig. 5.5B). For both carp and minnow, no presporogonic stages in the kidney interstitium or in liver and spleen were observed. The results obtained by histological examination are summarized in Table 5.3 for each single fish.

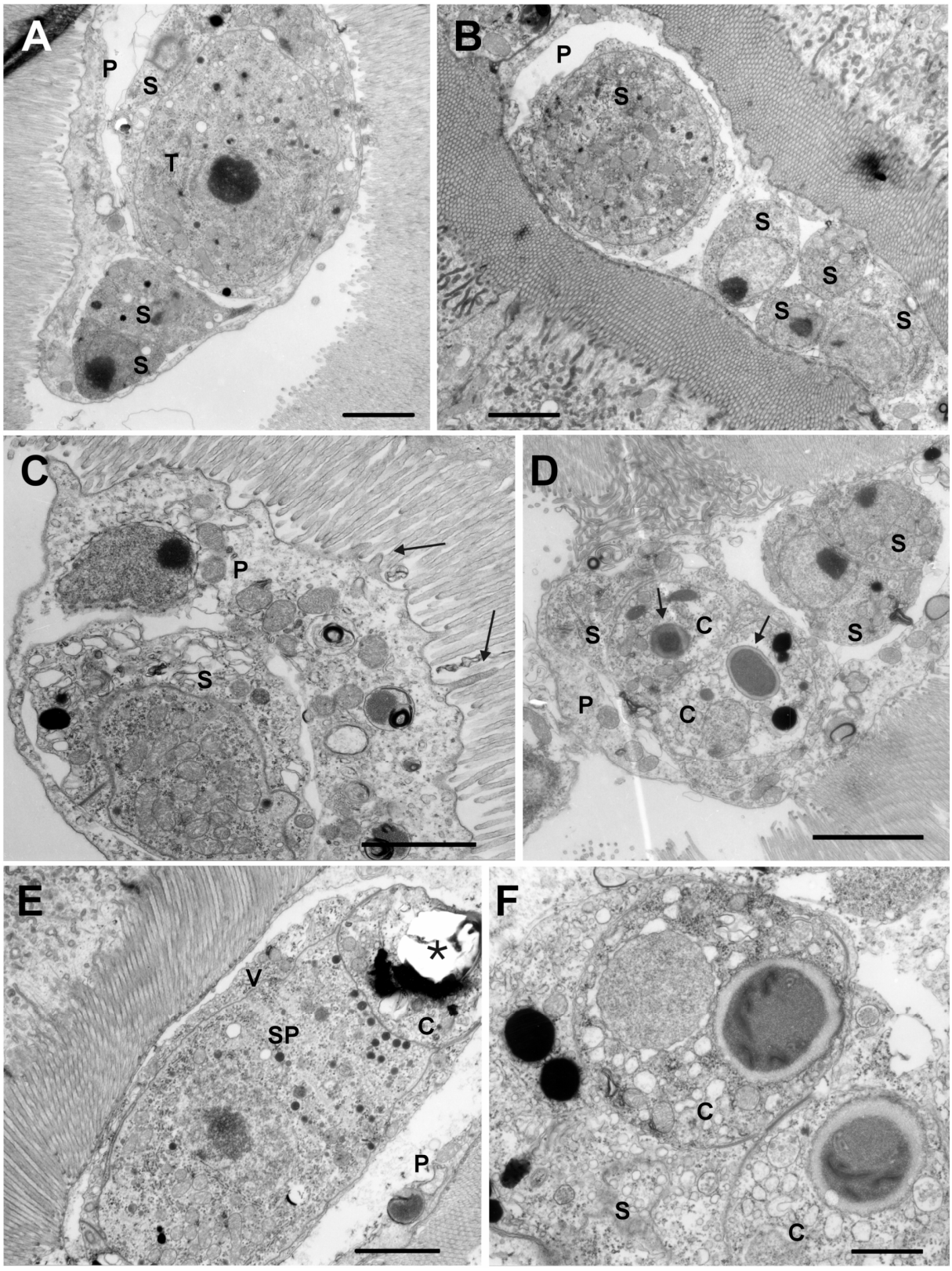


**Figure 5.4:** Electron micrographs of malacosporean stages in kidney tubules of carp at 8 wpe. **A**. Pseudoplasmodium: primary cell (P) enclosing secondary (S) and tertiary (T) cells. Bar: 2 µm. **B**. Pseudoplasmodium with 5 secondary cells (S) visible within the primary cell (P). Bar: 2 µm. **C**. Malacosporean stage with primary (P) and secondary cell (S). Note pseudopodia-like processes can be seen reaching between the microvilli of the tubular epithelium (arrows). Bar: 2 µm. **D**. Two capsulogenic cells (C) with polar capsules (arrows) inside a secondary cell (S). Two more secondary cells without tertiary cells. Bar: 3 µm. **E**. Maturing

spore with sporoplasm (SP) and one capsulogenic cell (C). The polar capsule has broken out of the section (asterisk). Valve cells (V) surrounding the sporoplasm and a part of the capsulogenic cell. Bar: 2 µm. **F**. Capsulogenic cells (C) with polar capsules; turns of polar filament are visible. Bar: 1 µm.

**Table 5.3 Sequencing and histology results of sampled fish**

| wpe[a] | Species and Time-Point | Tissue | *PCR-Result* mala f/r | *PCR-Result* budd f/r | Best match in GenBank | Histology |
|---|---|---|---|---|---|---|
| 3 | Carp 1 | kidney | + | + | *B. allmani* (AJ937880) 97.8 % | n.d. [c] |
| | | liver | + | + | *B. allmani* (AJ937880) 97.8 % | |
| | | spleen | + | n.d[c]. | not sequenced | |
| | Minnow 1 | kidney | + | + | *B. plumatellae*[b] (AY074914) 99.5 % | n.d. [c] |
| | | liver | - | n.d[c]. | | |
| | | spleen | - | n.d[c]. | | |
| | Brown trout 1 | kidney | + | n.d[c]. | *T. bryosalmonae* (U70623) 99.6 % | n.d. [c] |
| | | liver | - | n.d[c]. | | |
| | | spleen | - | n.d[c]. | | |
| 4 | Carp 2 | kidney | + | + | *B. allmani* (AJ937880) 97.8 % | no stages found |
| | Minnow 2 | kidney | + | + | *B. plumatellae*[b] (AY074914) 99.5 % | few intratubular stages, only single cells |
| | Brown trout 2 | kidney | - | n.d[c]. | | n.d. [c] |
| 5 | Carp 3 | kidney | + | + | *B. allmani* (AJ937880) 97.8 % | few intratubular stages in light microscopy |
| | Minnow 3 | kidney | - | n.d[c]. | | n.d. [c] |
| | Brown trout 3 | kidney | - | n.d[c]. | | n.d. [c] |
| 8 | Carp 4 | kidney | + | + | *B. allmani* (AJ937880) 97.8 % | numerous tubular sporogonic stages with polar capsules in light and electron microscopy |
| 10 | Carp 5 | kidney | + | | *B. allmani* (AJ937880) 97.8 % | few tubular stages and spores in light microscopy |
| 18 | Carp 6 | kidney | + | | *B. allmani* (AJ937880) 97.8 % | few tubular stages in light microscopy |

[a]wpe: weeks post exposure to infected bryozoans; [b]Sequences obtained from minnow were 100 % identical to the one from *Buddenbrockia* sp. in *P. repens*; [c]n.d.: not determined; +: positive; - negative PCR-result. Note: no parasite stages were detected by histology in spleen and liver of fish sampled after 3 wpe.

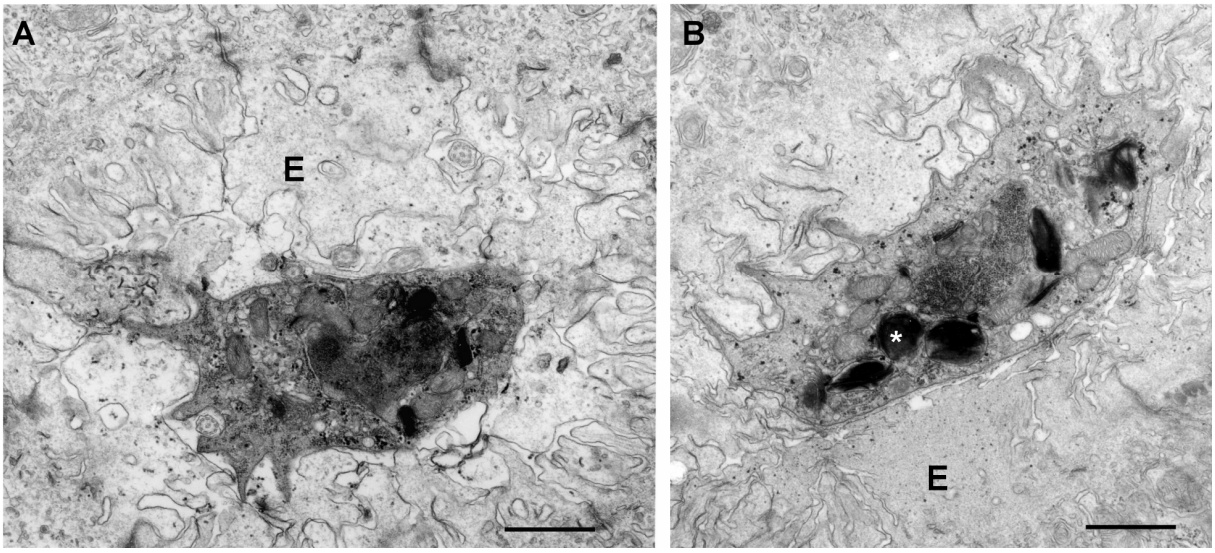


**Figure 5.5:** Electron micrographs of malacosporean stages in kidney tubules of minnow. **A**. Tubular single-cell parasite stage. Pseudopodia-like processes enclose host material. E: epithelial cells of kidney tubule. Bar: 1 µm. **B**. Tubular stages with vacuoles filled with stacks of electron dense material (asterisk). E: epithelial cells of kidney tubule. Bar: 1 µm.

## 5.4 Discussion

Prior to this study, developmental stages of *Buddenbrockia* species have only been described from the bryozoan host. Voronin (1993) and Voronin & Chernysheva (1993) provided some indication that malacosporeans, other than *Tetracapsuloides bryosalmonae*, parasitize fish by reporting malacosporean-like developmental stages in endothelial and pillar cells of gills, brain and kidney of common carp. The laboratory infection experiments conducted in the present study provide the first reliable evidence that cyprinid fish can act as a vertebrate host for two species of the genus *Buddenbrockia*. The infection experiment and molecular analyses showed that the worm-like parasite in the bryozoan *Plumatella repens* was able to infect minnow and is genetically identical to *Buddenbrockia plumatellae*. As the attempts to transmit this parasite from infected to non-infected bryozoans in the laboratory had failed previously (Tops *et al.* 2004), the finding of a susceptible fish host for *B. plumatellae* might be the key to elucidate the complete life-cycle of this parasite.

The sequences obtained from infected carp differed considerably from those detected in minnows and the malacosporean sequences in GenBank. Most similar was *B. allmani* (97.8 % 18S rDNA sequence identity), but this species is known to parasitize the bryozoan *Lophopus crystallinus* (Canning *et al.* 2007), which was not present in the sample of bryozoans used for

fish infection in the present study. This indicates that the malacosporean parasite from carp is likely a yet undescribed species.

While *T. bryosalmonae* is capable of infecting a wide variety of salmonids (Hedrick *et al.* 1993), the *Buddenbrockia* spp. investigated in the present study seemed to be more specific for their fish hosts, though only 2 minnows were infected and a mixed infection of the fish with more than one malacosporean can not be ruled out completely. Nevertheless, detection of the same malacosporean in all 6 exposed carp and not in minnow or brown trout provides at least some evidence that this malacosporean is more infective for carp than for the other two species tested. The infection with the same malacosporean detected in carp from two other locations and the late sporogonic stages found in the kidney tubules of infected carp provide evidence that this fish species might be the appropriate host for the undescribed *Buddenbrockia* sp. found in the present study. A bryozoan-derived stage of the carp-infective *Buddenbrockia* sp. was not observed, probably because of low prevalence of overt infections in the bryozoans collected from the field. *Buddenbrockia* spp. were found repeatedly in colonies of *P. repens* and morphological and size differences between spores from worm-like malacosporeans observed in *P. repens* have been reported. Morris *et al.* (2002a) found ornamented spores with a mean size of 19.0 µm, whereas the spores observed in the study of McGurk *et al.* (2006b) were spherical and measured 17.7 µm in diameter. These findings indicate the presence of at least two more *Buddenbrockia* species parasitizing this bryozoan. Therefore it was tested in the present study, if *P. repens* might be the host for the carp-malacosporean. However, no successful transmission from infected carp to bryozoans could be proven so far. Statoblast-raised *P. repens* were tested positive for malacosporean DNA by PCR after 3 weeks of cohabitation with infected carp. This shows, that either parasite DNA or possibly spores were released from fish and attached to the outer surface of bryozoans, were taken up with food particles, or penetrated the zooids. As the carp-cohabitated bryozoans sampled at later time points were all negative by PCR and no overt malacosporean infection developed in the exposed colonies, it is most likely that the *Buddenbrockia* sp. infectious for carp is specific to another species of bryozoans. Spores either could not enter *P. repens* or penetrated stages were destroyed by host defence mechanisms of the bryozoans. *P. fruticosa* or *Cristatella mucedo* were also found in the respective pond. Malacosporean infections had already been detected in the latter species as sac-like *Buddenbrockia* sp. (Canning & Okamura 2004; Tops *et al.* 2005). To our knowledge, no malacosporeans have been found in colonies of *P. fruticosa* so far. One of those species might be the host for the malacosporean detected

in carp. Also, some aquatic invertebrates were present on substrata and between the bryozoan colonies and could not be removed completely. It was shown that *T. bryosalmonae* requires only bryozoans and fish for completion of its life-cycle, but it is possible that other invertebrates may be required as intermediate hosts in the life-cycles of other malacosporeans. Further investigations are needed to find the invertebrate-host for the carp-malacosporean.

The findings of the present study show that at least three malacosporean species coexist in a single pond, as *T. bryosalmonae*, worm-shaped *B. plumatellae* and the undescribed *Buddenbrockia* sp. infecting carp were present. Taking into account the worldwide distribution of freshwater bryozoans and the various species in which malacosporean infections have already been detected (Canning & Okamura, 2004), it can be speculated that a substantially higher number of unknown malacosporean parasites must exist.

The sporogonic stages found in histological sections of infected carp kidneys were similar to stages described previously for *T. bryosalmonae* (see Hedrick *et al.* 1993 for review). Also, the ultrastructure of the luminal stages and the formation of spores in a primary cell (pseudoplasmodium) resembled to a great extent the development of *T. bryosalmonae* in fish kidney (Kent & Hedrick 1986, Morris & Adams 2008). It was observed that the capsulogenic cells form inside of a secondary cell, but the fate of the further four secondary cells found in the pseudoplasmodia is not clear. Morris & Adams (2008), reported similar cells in pseudoplasmodia of *T. bryosalmonae* where they form the valves of the spore and it is likely that this is also the case for the carp parasite. The origin of the sporoplasm remains unknown. Morris & Adams (2008) observed a sporoplasmogenic cell developing from the cell doublet to be forming the capsulogenic cells. This was not found in the present study as no intermediate stages were observed within this part of the developmental sequence. A schematic illustration of the spore formation of malacosporeans in the fish host according to the results of Morris & Adams (2008) and the present study is given in figure 5.6. Also, the timing of the development of the carp infecting malacosporean was similar to what was described in previous studies for *T. bryosalmonae*. Kent & Hedrick (1986) detected first intratubular stages of *T. bryosalmonae* in rainbow trout kept at 15 to 18 °C at 7 wpe, which was later found to be identical for brown trout (Morris & Adams 2008). Few intratubular stages in kidney of carp were found already after 5 wpe in the present study, but the peak of intratubular development and sporogony seemed to be at 8 wpe. Little information could be obtained about the development of the minnow parasite, as only a few single parasite cells were found. Further studies are under way to determine whether minnows are appropriate hosts for *B. plumatellae*

and to describe the development of this parasite in the fish host in more detail. Surprisingly, presporogonic stages could neither be observed by light and electron microscopy in organs of *Buddenbrockia*-infected carp nor minnow, therefore the proliferative phase of these parasites remains enigmatic. The malacosporean-like intracellular stages in common carp observed by Voronin (1993) and Voronin & Chernysheva (1993) might represent such early stages of a *Buddenbrockia* sp. that were not observed in the present study. An aim for future studies will be to describe the complete development of this carp malacosporean in fish, including the presporogonic stages and the cellular details of sporogony.

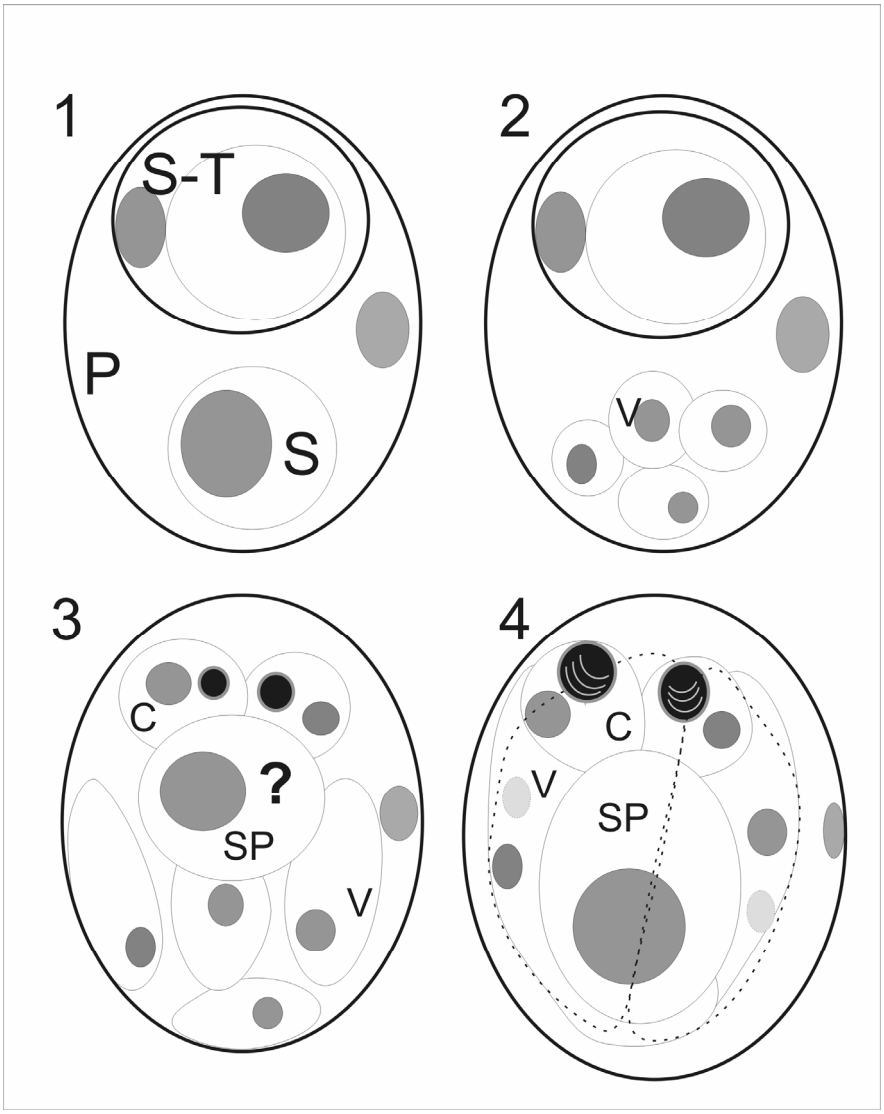


**Figure 5.6:** Schematic representation of the sporogonic sequence of malacosporeans according to Morris & Adams (2008) and results of the present study. Cells are white and nuclei are shaded grey. **1**. Pseudoplasmodium: primary cell (P) enclosing two secondary cells (S), one of them with tertiary cell (S-T-doublett). **2**. Secondary cell forms 4 valvogenic cells (V). **3**. Valvogenic cells (V) enclose sporoplasmogenic cell (SP; origin not clear) and capsulogenic cells (C) with precursors of polar capsules (black circles). **4**. Maturing spore, still enclosed by primary cell. Valve cells (V) enclose sporoplasmogenic (SP) and capsulogenic cells (C), which contain polar capsules.

Phylogenetic analysis did not fully clarify the relationship of the different *Buddenbrockia* spp. However, the results support the findings of Tops *et al.* (2005) and Jiménez-Guri *et al.* (2007b) that sac-shaped and worm-shaped *B. plumatellae* belong to two different clades. The placement of the undescribed *Buddenbrockia* sp. from *Fredericella sultana* and *B. allmani* (Tops *et al.* 2005, Canning *et al.* 2007) remained unclear. The malacosporean found in carp in the present study was placed as a sister-group to the *B. plumatellae*-clade, but the low node-probability makes this placement questionable. As common carp were introduced to Europe from Asia in medieval times, the parasite might have been imported too, or a European *Buddenbrockia* sp. adapted to the new fish host. A screening of indigenous cyprinids for malacosporean infections and phylogeographic studies are required to clarify history and origin of this parasite.

The results of the present study suggest that malacosporean parasites of the genus *Buddenbrockia* possess a life-cycle involving a fish host, similar to *T. bryosalmonae*. According to the sequence data, there may exist a further *Buddenbrockia* species to those already described, further enhancing diversity of the class Malacosporea. Infection trials with more fish and different bryozoan species under controlled laboratory conditions, and more detailed molecular and morphological examinations are planned to explain the findings of the present study. Another aim of future studies will be to find the susceptible fish species that can transmit malacosporean parasites of the genus *Buddenbrockia* to bryozoans.

# 6. GENERAL DISCUSSION

Establishment of a laboratory cycle of myxozoans was a prerequisite for advances in research on various species of this group, like for example *Myxobolus cerebralis*, an economically important parasite of salmonid fish. In the present study, a similar laboratory model was established for *Tetracapsuloides bryosalmonae*, which allowed research and experimental studies on this parasite independent from the seasonality of the disease in the field and the low prevalence of malacosporean infections detected in bryozoans in their natural habitats (Anderson *et al.* 1999b, Okamura *et al.* 2001, Okamura & Wood 2002). With a laboratory culturing system it was possible to obtain high spore numbers from infected bryozoans, thereby allowing controlled experimental infection of fish. By raising bryozoans from their durable stages, the statoblasts, parasite-free colonies could be obtained and used to test the capability of various fish species to transmit malacosporeans parasites.

The life-cycle of *T. bryosalmonae* was puzzling for a long time and Morris & Adams (2006a) were the first to prove that a fish host is essential for this parasite, by transmitting the disease from infected brown trout to the bryozoan *Fredericella sultana*. This result could be confirmed by transmission experiments in the present study. Additionally, infected brook trout (*Salvelinus fontinalis*) were shown to be able to transmit the parasite to bryozoans, while rainbow trout (*Oncorhynchus mykiss*) and grayling (*Thymallus thymallus*) were not. In former studies, sporogonic stages and spores were also found in rainbow trout infected in North America (Kent & Hedrick 1986, Hedrick *et al.* 2004), but not, if they were infected in Europe (Bucke *et al.* 1991). As a consequence of these findings, the existence of distinct European and American strains of *T. bryosalmonae* was proposed, and a recent study on the phylogeography of this parasite provides further support for this idea (Henderson & Okamura 2004). A variety of parasite strains, differing in their range of fish hosts was found for example for the acanthocephalan *Leptorhynchoides thecatus* and the cestode *Ligula intestinalis* (Steinauer *et al.* 2007, Štefka *et al*. 2009). To further clarify this issue for *T. bryosalmonae*, it should be attempted to infect species of the genera *Salvelinus*, *Salmo* and *Oncorhynchus* with

*T. bryosalmonae*-strains from Europe and North America and to compare susceptibility, parasite development, spore production, and infectivity of these spores for bryozoans.

The results of the present study provided strong evidence that the gill is the main portal of entry to the fish host for *T. bryosalmonae* and this entry locus is the first mechanical and immunological barrier for the sporoplasms. Additionally, the movement of sporoplasms of *T. bryosalmonae* could be shown *in vitro* for the first time. This knowledge will facilitate the investigation and comparison of the penetration efficiency of *T. bryosalmonae* spores to different fish hosts. The ability to attach to fish is a prerequisite for myxozoans to be able to complete their development in this host. As suggested by Kallert *et al*. (2005, 2009), an unspecific reaction behaviour of the actinospore-stage would be advantageous for these parasites to exploit new host species. Possibly, the finding of PKD-stages in pike from the field in previous studies (Seagrave *et al.* 1981, Morris *et al.* 2000a) indicates the adaptation of regional strains of *T. bryosalmonae* to this fish species. The unsuccessful infection-trials with pike in the present study might be due to an incompatible pair of host and parasite strains. It is not known what prevents myxozoan spores from infecting incompatible hosts. Kallert *et al*. (2009) could show unspecific attachment of *M. cerebralis* actinospores, but it was observed that the majority of sporoplasms did not enter a non-susceptible host. Either sporoplasms can not enter the fish efficiently or attached stages are eliminated quickly by mucus compounds and early phase immune response. Future research is needed to investigate the adaptation of myxozoan lineages to different hosts, and the role of host-switching for speciation of myxozoans.

Also, intra-specific differences of the fish host lead to varying levels of susceptibility to parasites. In the present study, two strains of rainbow trout were identified which seemed to be more resistant to *T. bryosalmonae* compared to the other strains tested and brown trout. It was discussed above (section 4.4) that early phase response is likely to be the cause of these observed differences in resistance. The antibody-response against myxozoans is slow (Sitjà-Bobadilla 2008). Therefore, it can be assumed that the acquired immune response will be a more important factor differentiating fish strains in their susceptibility to the parasite in later phases of the infection. In the end, a combination of innate and adaptive immune response will contribute to resistance or susceptibility of the host (Jones 2001). The immune response of the host might also interfere with developmental process and differentiation of parasite cells. Therefore, the above mentioned inability of *T. bryosalmonae* to form mature spores in certain fish species might be an evidence for the incompatibility of host and parasite, possibly

caused by the immune response of the fish. This might have also been the case for the *Buddenbrockia*-infected carp in the present study, where maturing spores were observed in the kidney tubules (section 5.3.5), but bryozoans could not be overtly infected by cohabitation with these fish.

Evolution of parasites is driven mainly by colonization of new hosts or co-evolution of host and parasite (Poulin & Morand 2000). It was discussed in section 4.4 that the observed resistance of one strain of rainbow trout in the present study might be caused by co-adaptation of these fish with *T. bryosalmonae*. A similar case of parasite-host co-adaptation was observed in previous infection experiments with two strains of three-spined sticklebacks (*Gasterosteus aculeatus*) and the eye fluke (*Diplostomum pseudospathaceum*). One of these fish-strains was found to be less susceptible to the parasite than the other, which was explained by the adaptation of one stickleback population to a higher parasite load due to their habitat, causing the increased resistance against eye flukes (Kalbe & Kurtz 2006). If the same is the case for *T. bryosalmonae*-resistant rainbow trout, the genetic basis of the resistance has to be identified. This would provide a starting point for breeding experiments to obtain fish strains that can be used for aquaculture in regions with PKD-problems.

Before the present study, malacosporeans other than *T. bryosalmonae* were only known from bryozoan hosts. The striking finding of *Buddenbrockia* spp. infecting cyprinid fish showed that *T. bryosalmonae* is not the only fish-infecting malacosporean. These results open the field for research on fish-bryozoan life-cycles of this group. Most likely two-host life-cycles are common in this group of bryozoan-specific myxozoans. Nevertheless, the full life-cycle including infection of bryozoans with stages derived from infected fish has still to be proven. As the infection of fish by *Buddenbrockia* spp. was overlooked for such a long time, it is likely, that a much higher diversity of malacosporean parasites exists, probably not causing any severe symptoms in infected hosts.

Systematics of the Malacosporea have not been fully clarified yet. Obviously, the phylogenetic relationships of the *Buddenbrockia* spp. are hard to evaluate by 18S rDNA-sequence comparisons, due to the high similarity of these sequences. Also, morphological characteristics mostly require a detailed ultrastructural study of the parasite stages. Therefore, the phylogenetic placement of the carp-infecting species found in the present study and the question, if worm and sac-shaped forms of *B. plumatellae* belong to one species can not be fully clarified to date. Further studies are required that include protein coding genes (Jiménez-Guri *et al.* 2007b) or defined areas of 18S and 28S rDNA (Bartošová *et al.* 2009) in molecular

phylogenetic analyses, in conjunction with life-cycle studies and investigation of host specificity to resolve the phylogeny of the Malacosporea. So far, all malacosporean stages found in the fish host (*T. bryosalmonae*, *Buddenbrockia* sp. detected in carp in the present study and possibly *B. plumatellae* detected in minnow) are kidney specific. It would be an interesting task to find out if other malacosporeans (e.g. *B. allmani* or the yet undescribed worm-like *Buddenbrockia* sp. found in *F. sultana*) also possess a fish host and if the target organ of those species is also the kidney. Additionally, the increasing number of known fresh-water-malacosporeans opens the field for speculations about marine ancestors of this group. As the Malacosporea are more ancient than the Myxosporea, it is likely that marine malaco-sporeans exist, also parasitizing bryozoans.

The results obtained in this thesis also raised several questions that will be starting points for future studies. It remains unknown which other salmonids besides the ones tested in the present study are capable of transmitting PKD to bryozoans. Also, the important issue of different geographically isolated strains of parasite, fish and bryozoan host remains to be addressed. Although the morphology and penetration of *T. bryosalmonae*-spores to the fish could be characterized, the further fate of parasite stages after penetration and before the proliferation in the kidney tissue is still not clear and has to be investigated. Mechanistic reasons for increased resistance of some trout strains to PKD require further research, same as the question about heritability of the resistance. To improve the knowledge about the Malacosporea, the next important step should be the search for further fish hosts and complete life-cycles of *Buddenbrockia* spp.

# 7. ZUSAMMENFASSUNG

Der Stamm Myxozoa gehört zu den Metazoa und umfasst ca. 2.000, meist fischparasitische Arten. Typisch für Vertreter dieses Phylums ist ein Wirtswechsel zwischen Wirbeltier und einem wirbellosen Wirt. Die Übertragung erfolgt dabei über Sporen, die infektiös für den jeweiligen Wirt sind. Die wichtigsten Merkmale der Gruppe sind die Zellvermehrung über Endogenie, das Fehlen von Zentriolen bei der Zellteilung, die Bildung von Sekundärzellen und die Bildung von vielzelligen Sporen in Plasmodien oder Pseudoplasmodien. Die Myxozoa lassen sich weiter unterteilen in die Klassen Myxosporea, die den größten Teil der Artenvielfalt dieser Gruppe ausmachen und die Malacosporea mit bis dato nur 3 beschriebenen Arten. Die Klasse Malacosporea ist die rätselhafteste und wahrscheinlich die ursprünglichste Gruppe innerhalb des einzigartigen Stammes Myxozoa. Es ist bekannt, dass diese Parasiten sowohl Fische als auch Süßwasserbryozoen infizieren, allerdings wurden die meisten Malacosporea bisher nur im Wirbellosenwirt beschrieben. Der vollständige Lebenszyklus ist lediglich für die Art *Tetracapsuloides bryosalmonae* bekannt. Dieser Parasit verursacht die „Proliferative Kidney Disease“ bei Salmoniden, die in der Forellenaquakultur zu hohen Verlusten führen kann und möglicherweise auch die Populationen wildlebender Bachforellen (*Salmo trutta*) bedroht. Der Parasit entwickelt sich hierbei zunächst im Interstitialgewebe der Niere der Fische und kann dort zu granulomatösen Veränderungen führen. Später wandern die Stadien durch das Tubulusepithel in die Nierentubuli, wo dann die Bildung der Sporen stattfindet. Diese Sporen werden über den Urin ins umgebende Wasser abgegeben, wo sie infektiös für Bryozoen sind. Der genaue Weg, über welchen die vom Fisch ausgeschiedenen Sporen in die Bryozoen gelangen, ist noch unklar. Die frühesten bekannten Stadien in den Bryozoen sind einzellig und befinden sich angeheftet an der Wand der Leibeshöhle. Dort entwickeln sie sich zunächst und bilden später Aggregate von Zellen, die in der Leibeshöhlenflüssigkeit der Bryozoen flottieren. Aus diesen Aggregaten entstehen die sogenannten Sporensäcke, aus denen die eigentlichen Sporen (Bryozoa-Sporen) hervorgehen. Diese werden durch Aufreißen der Säcke frei und schließlich aus den Bryozoen ins Wasser abgegeben, wo sie dann Salmoniden infizieren können. Neben der Gattung *Tetracapsuloides*

mit nur einer Art umfassen die Malacosporea auch noch die Gattung *Buddenbrockia* mit zurzeit zwei beschriebenen Arten. *Buddenbrockia* spp. sind bisher nur aus dem Bryozoenwirt bekannt. Dort bilden sie entweder sackartige Stadien, ähnlich denen von *T. bryosalmonae* oder wurmartige, bewegliche Formen. Kenntnisse über Wirtsspektrum, Übertragungsmechanismen und innerartliche Unterschiede in der Empfänglichkeit gegenüber Malacosporea-Erregern sind bislang rar. Daher war es das Ziel der vorliegenden Arbeit Biologie und Lebenszyklen einiger Malacosporea-Parasiten intensiver zu untersuchen. Zunächst wurde eine Laborkultur von Süßwasserbryozoen etabliert, die für die Erhaltung des Parasitenzyklus im Labor notwendig war. Dazu wurden die Tiere mit ihrem natürlichen Substrat, was typischerweise ein Stück totes Holz oder eine Wurzel ist, an konventionelle Plastikpetrischalen geklebt. Diese wurden dann in geeigneten Halterungen mit der Oberseite nach unten in Eimern mit entchlortem Wasser gehalten. Die Fütterung erfolgte über eigens gezüchtete Algenkulturen. Erst die Sporen, die aus Malacosporea-infizierten, kultivierten Bryozoen gewonnen werden konnten, ermöglichten Transmissionsversuche unter kontrollierten Bedingungen. Zudem wurden parasitenfreie Bryozoenkolonien aus den Dauerstadien, den sogenannten Statoblasten, herangezogen, indem diese unter geeigneten Bedingungen zur Entwicklung gebracht wurden.

Es ist seit langem bekannt, dass die meisten Salmonidenarten empfänglich für die Infektion mit *T. bryosalmonae* sind. Auch in den Nieren von Hechten (*Esox lucius*) konnten in früheren histologischen Untersuchungen Entwicklungsstadien ähnlich diesem Parasiten gefunden werden. In einer vorangegangenen Arbeit gelang jedoch die Übertragung dieses Parasiten auf Bryozoen im Labor nur mittels infizierter Bachforellen. Daher wurde in der vorliegenden Arbeit mit Hilfe von Infektionsversuchen untersucht, ob neben Bachforellen möglicherweise auch andere Fische geeignete Wirte für *T. bryosalmonae* darstellen. Verschiedene in Frage kommende Fischarten wurden unter Laborbedingungen mit dem Parasiten infiziert, indem alle zusammen in einem Aquarium für 2 Wochen gegenüber Kolonien infizierter Bryozoen exponiert wurden. Lediglich die Hechte mussten separat infiziert werden, um Prädation der anderen Fische zu vermeiden. Um den Erfolg der Infektion zu testen wurde ein Fisch aus jeder Gruppe seziert und eine Probe der Niere mittels *T. bryosalmonae*-spezifischer PCR untersucht. Dabei wurden alle untersuchten Fische positiv getestet. Die Sporogenese im Fisch setzt normalerweise nach ca. 7-8 Wochen nach der Infektion ein. Daher wurden die *T. bryosalmonae*-infizierten Fische 8 Wochen nach Beginn der Infektion mit parasitenfreien Laborzuchten von Bryozoen für 6 Monate zusammen gehalten. In früheren Arbeiten wurde

zum Teil die Vermutung geäußert, dass die Dauerstadien der Bryozoen Malacosporea-Stadien enthalten, welche damit die Infektion an die nächste Generation weitergeben können. Um den parasitenfreien Status der SPF Bryozoenkolonien sicherzustellen war es daher notwendig Proben von Statoblasten der verwendeten Bryozoenart mittels PCR zu testen. Trotz einer großen Anzahl an untersuchten Statoblasten konnten aber keine Hinweise auf eine kryptische Infektion dieser Stadien gefunden werden. In den Versuchen der vorliegenden Arbeit konnte des Weiteren nachgewiesen werden, dass neben Bachforellen auch Bachsaiblinge (*Salvelinus fontinalis*) *T. bryosalmonae* auf Bryozoen übertragen können. Bei den gegenüber diesen beiden Arten exponierten Bryozoenkolonien zeigten sich nach 5 Wochen die typischen sack-artigen Stadien von *T. bryosalmonae* in der Leibeshöhle. Im Gegensatz dazu lösten infizierte Regenbogenforellen (*Oncorhynchus mykiss*) und Äschen (*Thymallus thymallus*) keine Infektion bei den Bryozoen aus. Hechte konnten in diesen Versuchen nicht infiziert werden, da molekularbiologisch, mittels *T. bryosalmonae*-spezifischer PCR, keine Parasitenstadien in der Niere nachgewiesen werden konnten. Dies lässt darauf schließen, dass die in früheren Arbeiten bei Hechten gefundenen Entwicklungsstadien möglicherweise auf eine Infektion mit einem anderen Malacosporea-Parasiten zurückzuführen waren. Der Nachweis der von den Fischen ausgeschiedenen Sporen über Filtration des Aquarienwassers schlug fehl, vermutlich wegen deren geringer Größe, der zeitlichen Fokussierung der Ausscheidung und der geringen Zahl der im Fisch produzierten Sporen. In histologischen Schnitten, die immunhistochemisch mit einem *T. bryosalmonae*-spezifischen Antikörper gefärbt wurden, konnten sporogonische Stadien nur in den Nieren von Bachforellen gefunden werden, nicht jedoch bei Bach-saiblingen. Dies deutet wiederum darauf hin, dass Sporen nur über einen relativ kurzen Zeitraum von einigen Wochen ausgeschieden werden und bei den untersuchten Saiblingen zu diesem Zeitpunkt keine reifen Stadien in den Nierentubuli vorhanden waren.

Da *T. bryosalmonae* eine große Vielzahl an Fischarten infizieren kann, ist anzunehmen, dass bei diesem Parasiten ein unspezifischer Modus der Wirtsinvasion vorhanden ist. Bisher sind über die Anheftung und die Penetration von Sporen der Malacosporea bei Kontakt mit dem Fischwirt nur wenige Details bekannt. Daher sollte in der vorliegenden Arbeit geklärt werden, welche morphologischen Eigenheiten die von den Bryozoen freigesetzten Sporen von *T. bryosalmonae* (Bryozoa-Sporen) von denen anderer Myxozoenarten unterscheiden, wie die Anheftung an und die Penetration in den Fischwirt ablaufen und wo die Eintrittspforte in den Fischkörper liegt. Um diese frühen Stadien des Lebenszyklus ultrastrukturell darzustellen, wurden Infektionsversuche und *in vitro* Experimente mit Bryozoa-Sporen von *T.*

*bryosalmonae* durchgeführt. Die notwendigen Sporen wurden aus dem Laborzyklus des Parasiten gewonnen. Um eine ausreichende Anzahl an Sporen zu erhalten, mussten Teile von Bryozoenkolonien, die Sporen oder reife sackartige Stadien enthielten, seziert werden. Für die chemische Aktivierung der Sporen wurde Forellenhautschleim-Homogenat verwendet, das zur mechanischen Stimulation zusammen mit der Sporensuspension einige Male in eine Pipettenspitze gesaugt wurde. Die Untersuchung der Reaktion der Sporen erfolgte mikroskopisch auf einem Objektträger. Zur Ermittlung der Eintrittspforte von *T. bryosalmonae* in den Fisch wurden SPF Regenbogenforellenbrütlinge gegenüber 1.000 bis 2.000 Bryozoa-Sporen des Parasiten in einem geringen Volumen Wasser exponiert. Dieser Versuch wurde mehrfach mit verschiedenen Inkubationszeiten zwischen 1 min und 60 min wiederholt. Die Fische wurden getötet und Kiemen sowie Stücke der Haut für Licht- und Transmissionselektronenmikroskopie fixiert. Zudem wurden Kiemen, ganze Fische und Sporensuspensionen für die Rasterelektronenmikroskopie verwendet. So konnte erstmals rasterelektronenmikroskopisch die Morphologie der Bryozoa-Sporen von *T. bryosalmonae* untersucht werden. Dabei wurde ersichtlich, dass die Polkapselzellen von den Hüllzellen umschlossen werden und nur eine Öffnung für das Polfilament erhalten bleibt, die wiederum von einem Pfropfen verschlossen ist. Die amöboide Bewegung der Sporoplasmen konnte durch die *in vitro* Aktivierungsversuche dargestellt werden. Nach der Aktivierung begannen die Sporoplasmazellen die Sporenhülle zu verlassen und bewegten sich langsam unter der Bildung von Pseudopodien. Bei der lichtmikroskopischen Auswertung der immunhistochemisch gefärbten histologischen Präparate konnten an den Kiemen sowohl angeheftete Sporen als auch penetrierende und bereits in die Kieme eingedrungene Stadien beobachtet werden, wobei die meisten Parasitenstadien 20 und 30 min nach Exposition gefunden wurden. Im Gegensatz dazu konnten in oder an der Haut keine Parasitenstadien gefunden werden. Dementsprechend deuten die ultrasturkturellen Befunde darauf hin, dass die Kiemen die Haupteintrittspforte für diesen Parasiten sind. Licht- und transmissionselektronenmikroskopisch konnte des Weiteren gezeigt werden, dass die Sporoplasmen von *T. bryosalmonae* in den Körper des Fisches direkt durch das Kiemenepithel eindringen und nicht durch Schleimdrüsenzellen, wie dies zum Beispiel bei *Myxobolus cerebralis* der Fall ist. Vermutlich auf Grund ungeeigneter Verarbeitungsmethoden der Proben konnten im Rasterelektronenmikroskop weder auf den Kiemen noch auf der Oberfläche der ganzen Fische Sporen nachgewiesen werden.

Nach der Penetration gelangt der Parasit höchst wahrscheinlich über das Blutgefäßsystem zum Zielorgan. Dort ist er dem Immunsystem des Wirtes ausgesetzt, weswegen die Anzahl

der eingedrungenen Stadien und die Wirtsreaktion gegen den Parasiten die Entwicklung und Vermehrung desselben beeinflussen. Genetisch bedingte Unterschiede bei verschiedenen Stämmen des Wirbeltierwirtes können sowohl den Erfolg der Penetration als auch die weitere Entwicklung des Parasiten beeinflussen. Diese Variabilität der Empfänglichkeit wurde in der vorliegenden Arbeit untersucht, da verlässliche Daten über intraspezifische Unterschiede der Reaktion von Fischen gegen Malacosporea-Infektionen bis heute fehlen. Zu diesem Zweck wurden vier Regenbogenforellenstämme und Bachforellen unter identischen Bedingungen mit *T. bryosalmonae* infiziert. Nach 2, 3 und 4 Wochen nach der Exposition der Fische wurden jeweils 8 Fische jedes Stammes/Art getötet, ihre Größe und Gewicht bestimmt und Proben der Niere für molekularbiologische und histochemische Untersuchungen genommen, um die Intensität der Infektion zu überwachen. Speziell für die Quantifizierung der Parasitenstadien wurde ein *T. bryosalmonae*-spezifischer quantitativer real-time PCR-Assay (q-RT-PCR) entwickelt. Dazu wurden anhand von Sequenzinformationen zu *T. bryosalmonae* und anderen Myxozoa in der GeneBank spezifische Primer konstruiert, die ein 166 bp langes Stück der 18S rDNA des Parasiten amplifizieren. Um eine Quantifizierung relativ zur Anzahl der Wirtszellen zu ermöglichen wurden bereits beschriebene Primer spezifisch für das insulin-like growth factor 1 (IGF 1)-Gen von Forellen verwendet. Die Reaktionsbedingungen für die Primer wurden optimiert und ihre Effizienz in Vorversuchen bestimmt. Die q-RT-PCR wurde mittels Sybr-Green Detektion durchgeführt. Die Auswertung der q-RT-PCR Daten der einzelnen Zeitpunkte zeigte, dass die verwendeten Bachforellen empfänglicher gegenüber dem Parasiten waren als alle Regenbogenforellenstämme. An den ersten beiden Probennahmezeitpunkten des Experiments, 2 und 3 Wochen nach Exposition, wiesen zwei der Regenbogenforellenstämme eine signifikant geringere Parasitenlast auf als alle anderen Gruppen, allerdings vermehrten sich bei diesen beiden Stämmen die Parasiten schneller, was am letzten Entnahmezeitpunkt 4 Wochen nach Exposition letztlich zur Angleichung der relativen Infektionsrate bei allen Regenbogenforellenstämmen führte. Mittels Immunhistochemie konnten Stadien von *T. bryosalmonae* in histologischen Schnitten detektiert werden, jedoch war ihre Anzahl gering, was unglücklicherweise eine quantitative Evaluierung der Parasitenlast unmöglich machte. Eine schwache negative Korrelation zwischen Größe der Fische und Parasitenlast konnte festgestellt werden. Insgesamt deuten diese Ergebnisse darauf hin, dass manche Forellenstämme in der Lage sind die Vermehrung des Parasiten nach den ersten Wochen zu bremsen, was möglicherweise auf eine effizientere antikörpergebundene Immunantwort zurückzuführen ist. Auffällig war bei diesen Ergebnissen, dass der

vergleichsweise *T. bryosalmonae*-resistente Regenbogenforellenstamm derjenige war, der sich in vorangegangenen Arbeiten als besonders empfänglich für einen anderen Myxozoen-parasiten, *M. cerebralis*, erwiesen hatte. Offensichtlich lässt also die Resistenz gegen eine Myxozoenart nicht auf die Empfänglichkeit gegenüber anderen Vertretern dieser Gruppe schließen.

Neben *T. bryosalmonae* sind noch zwei weitere Malacosporea-Arten beschrieben, die ausnahmslos der Gattung *Buddenbrockia* zugeordnet werden. Kenntnisse über Lebenszyklen dieser Arten sind unvollständig, da diese bisher lediglich als Stadien im Bryozoenwirt beschrieben worden waren. In der vorliegenden Arbeit sollte die Frage geklärt werden, ob Malacosporea der Gattung *Buddenbrockia* ebenso einen Fischwirt in ihrem Lebenszyklus besitzen, wie dies bei *T. bryosalmonae* der Fall ist. Zu diesem Zweck wurden verschiedene Bryozoenarten aus einem naturnahen Teich gesammelt und auf Infektionen mit den sack- oder wurmartigen Malacosporeastadien hin untersucht. Über infizierte Bryozoen der Art *Plumatella repens* konnten mittels Kohabitationsversuchen parasitenfreie Karpfen (*Cyprinus carpio*) und Elritzen (*Phoxinus phoxinus*) infiziert werden. Diese bisher völlig unbekannten Parasitenstadien konnten in den Nieren beider Fischarten nachgewiesen werden. Durch Sequenzvergleich eines 1.600 bp langen Abschnitts der 18S rDNA konnte der Elritzenparasit als *Buddenbrockia plumatellae* identifiziert werden (99,5 % Übereinstimmung der Sequenzen), wohingegen es sich bei dem Karpfenparasiten höchst wahrscheinlich um eine bisher unbeschriebene Art der Gattung *Buddenbrockia* handelte. Die höchste Übereinstimmung der 18S rDNA von letzterem Parasitenisolat konnte für *B. allmani* gefunden werden und lag bei 97,8 %. Ebenfalls exponierte Bachforellen wurden nicht mit den *Buddenbrockia* spp. infiziert. Einzelne der infizierten Karpfen und Elritzen wurden zu verschiedenen Zeitpunkten nach der Infektion getötet und Proben der Niere entnommen. Mittels licht- und elektronenmikroskopischer Untersuchungen der Nierenproben dieser Fische konnten Parasitenstadien in den Nierentubuli dargestellt werden. Bei den Elritzen konnten nur einzelne Parasitenzellen beobachtet werden, wohingegen in Schnitten der Nierentubuli von Karpfen zahlreiche, mehrzellige sporogonische Stadien gefunden wurden. Acht Wochen nach der Exposition gegenüber den Bryozoen konnten bei den Karpfen die meisten Stadien nachgewiesen werden. Diese Entwicklungsstadien bestanden aus Pseudoplasmodien mit Sekundär- Tertiäzellen unterschiedlicher Differenzierung und wiesen eine auffällige Ähnlichkeit mit den Fischstadien von *T. bryosalmonae* auf. Da von den Elritzen nur 3 Exemplare zur Verfügung standen, konnten von dieser Art nur Proben bis 5 Wochen nach

Exposition genommen werden. Bisher konnten weder bei den Karpfen noch bei den Elritzen präsporogonische Stadien gefunden werden, was darauf hindeutet, dass diese möglicherweise in einem anderen Organ zu finden sein müssen. Die Kohabitation von parasitenfreien, im Labor gezüchteten Bryozoen mit den *Buddenbrockia*-infizierten Karpfen führte zu keinem offenen Ausbruch der Infektion, lediglich parasitäre DNA konnte an Teilen der Kolonie detektiert werden. Dementsprechend muss der vollständige Lebenszyklus dieses Parasiten noch etabliert werden, um auch die Infektion von Bryozoen mittels infizierter Fische zweifelsfrei nachweisen zu können. Anhand der erhaltenen 18S rDNA Sequenzinformationen wurde eine phylogenetische Analyse der heute bekannten *Buddenbrockia* spp. durchgeführt. Dazu wurde ein multiples Alignment erstellt und mittels Bayescher Verfahren ein hypothetischer Stammbaum der Arten ermittelt. Dieser stützte die bereits vermutete Aufteilung der Art *B. plumatellae* in zwei Linien, jedoch konnten die Karpfen-infizierenden Malacosporea nicht zufriedenstellend eingruppiert werden, da die Unterschiede in den zur Verfügung stehenden Sequenzen zu gering waren, um eine höhere Auflösung der phylogenetischen Analyse zu ermöglichen.

Die Ergebnisse der vorliegenden Arbeit haben in entscheidendem Maße die Kenntnisse über das Wirtsspektrum, Wirt-Parasit-Interaktion und die Entwicklungszyklen von verschiedenen, auch mutmaßlich neuen, Malacosporea-Arten erweitert. Dies war möglich mit Hilfe der Bryozoenkulturen und des im Rahmen dieser Arbeit etablierten Laborzyklus von *T. bryosalmonae*. Das Ergebnis, dass einige suszeptible Salmoniden den Parasiten auf Bryozoen übertragen können und andere nicht, erhärtete die Vermutung, dass es einen europäischen und einen nordamerikanischen *T. bryosalmonae*-Stamm mit jeweils unterschiedlichem Spektrum an kompatiblen Wirten zu geben scheint. Weitere Studien sind wünschenswert, um diesen evolutionsbiologisch interessanten Aspekt der Lebensweise dieses Parasiten zu klären. Die Ergebnisse der Untersuchungen der vorliegenden Arbeit zur Invasion von *T. bryosalmonae* in den Fisch trugen entscheidend zur weiteren Aufklärung der frühen Infektionsprozesse der Malacosporea bei. Erstmals konnte die amöboide Bewegung der Sporoplasmen von *T. bryosalmonae in vitro* gezeigt und das Eindringen des Parasiten in die Kiemen der Fische histologisch dargestellt werden. Diese Ergebnisse ermöglichen für die Zukunft die weitergehende Untersuchung von Wirts- und Gewebespezifität der Malacosporea. Ebenso ergaben sich Hinweise auf Resistenzunterschiede verschiedener Regenbogenforellenstämme gegenüber *T. bryosalmonae*, wenngleich die Mechanismen dieser Unterschiede noch geklärt werden müssen. Im Zusammenhang mit den Versuchen zur Wirtsspezifität des Parasiten lässt sich

vermuten, dass Coevolution von Parasit und Wirt sowohl zu Unterschieden in der Empfänglichkeit, als auch der unvollständigen Entwicklung des Parasiten in nicht angepassten Wirten führen kann. Ein besonders spektakuläres Ergebnis dieser Arbeit war die Entdeckung, dass manche *Buddenbrockia*-Arten ebenso wie *T. bryosalmonae* in der Lage sind Fische zu infizieren und sich in diesen bis zur Sporogenese zu entwickeln. Zu klären ist dabei noch die Übertragung vom infizierten Fisch auf die Bryozoen und die Frage, ob andere Arten der Gattung *Buddenbrockia* ebenfalls einen Fischwirt in ihrem Lebenszyklus benötigen.

# 8. SUMMARY

The Metazoan phylum Myxozoa comprises about 2,000 mostly fish-parasitic species. Typical for this group are life-cycles involving a vertebrate and an invertebrate host. Transmission from one host to the other is achieved by spores that are infectious for the respective host. The most important characteristics of the Myxozoa are multiplication by endogeny, lack of centrioles during cell division, secondary cell formation and formation of multicellular spores in plasmodia or pseudoplasmodia. The Myxozoa can be further divided in the classes Myxosporea and Malacosporea. Most myxozoan species belong to the Myxosporea, while up to now only 3 malacosporean species are described. The class Malacosporea is the most enigmatic and probably most ancient group within the unique phylum Myxozoa. These parasites are known to infect fish and freshwater bryozoans, though most malacosporeans have been described only in the invertebrate host. The only complete malacosporean life-cycle is known for *Tetracapsuloides bryosalmonae*, the parasite causing proliferative kidney disease (PKD) in salmonid fish. This disease can lead to high losses in aquaculture and might also threaten wild populations of brown trout (*Salmo trutta*). The parasite initially develops in the interstitial tissue of the fish kidney and can cause granulomatous changes. Later, these stages migrate trough the tubular epithelium into the kidney tubules, where spores are formed. These spores are released to the surrounding water by the urine, where they are infective for bryozoans. The exact route where the spores released by the fish host enter the bryozoans is not clear yet. The earliest known parasite stages in the bryozoans are unicellular and are attached at the inside of the peritoneal wall. Later on, they develop and form aggregates of cells that float in the coelomic fluid of the body cavity of the bryozoans. These aggregates grow further and form hollow spore sacs inside of which the spores (bryozoa-spores) are formed. After maturation, spores are released by rupture of the sacs and are shed to the water, where they infect new fish hosts. Besides the genus *Tetracapsuloides* with only a single species, the Malacosporea also contain the genus *Buddenbrockia* with two described species. *Buddenbrockia* spp. are known only from the bryozoan host where they either form sac-like stages similar to *T. bryosalmonae* or worm-like, motile stages. Knowledge about host

spectrum, transmission mechanisms and intra-specific differences in susceptibility to malacosporean parasites are scarce. Therefore, it was the aim of the present study to investigate biology and life-cycles of some malacosporeans more intensively. First, a culture of freshwater bryozoans, necessary for the maintenance of the parasite in the laboratory, was established. To this end, the bryozoan colonies collected from the field were glued to conventional Petri-dishes on their natural substrate, typically pieces of dead wood or roots. These were placed upside down in suitable racks in buckets filled with dechlorinated water. To feed the colonies, several species of algae had to be cultured. The spores, obtained from the Malacosporea-infected, cultivated bryozoans allowed transmission experiments under controlled laboratory conditions. Additionally, parasite free bryozoan colonies were raised from the durable stages of bryozoans, so-called statoblasts, by germinating them under the appropriate conditions.

It is long known, that most salmonid species are susceptible for infection with *T. bryosalmonae*. Also in kidneys of pike (*Esox lucius*) developmental stages similar to this parasite were detected in previous studies. So far, the transmission of the parasite to bryozoans in the laboratory was achieved only with brown trout. Therefore, infection trials were conducted in the present study, to investigate, if other fish species might be adequate hosts for *T. bryosalmonae*. Different fish species of interest were infected with the parasite under laboratory conditions, by exposing all fish together in one aquarium for 2 weeks to colonies of infected bryozoans. Only the pike had to be infected separately to avoid predation. To test the success of infection, one fish of each group was dissected three weeks after beginning of exposure (wpe) and a sample of the kidney was tested by *T. bryosalmonae*-specific PCR. All fish were tested positive for the parasite. Normally, sporogenesis in the fish starts 7 to 8 weeks after infection. Hence, parasite free bryozoans were exposed to the *T. bryosalmonae*-infected fish starting 8 wpe for a period of 6 months. The possibility for presence of cryptic malacosporean stages in statoblasts of bryozoans was suggested in some previous studies. These stages would transmit the infection to the next Bryozoa-generation. To ascertain the parasite free status of the laboratory raised bryozoan colonies it was necessary to test the statoblasts of this bryozoan species for incorporated parasite stages by PCR. Although a large number of statoblasts was investigated, no evidence for cryptic infections of these statoblasts was found. The results of the infection experiments of the present study proved that besides brown trout also brook trout (*Salvelinus fontinalis*) can transmit *T. bryosalmonae* to bryozoans. In the body cavity of bryozoan colonies exposed to these two species, typical sac-

like stages of *T. bryosalmonae* were found after 5 wpe. In contrast, infected rainbow trout (*Oncorhynchus mykiss*) and grayling (*Thymallus thymallus*) did not cause an infection of bryozoans. Surprisingly, pike did not become infected at all in these experiments. No parasite stages were detected in the kidney of these fish, even by *T. bryosalmonae*-specific PCR. It can be speculated that the developmental stages found in pike in previous studies were other malacosporean parasites. The detection of spores released from fish by filtration of aquarium water failed, probably because of the small size, the temporal focused spore-release and the low number of spores produced in the fish. In histological sections stained immuno-histochemically with a *T. bryosalmonae*-specific antibody, sporogonic stages were found only in the kidney of brown trout but not in brook trout kidney. This again indicates that spores are released only during a short period of time and that the brook trout investigated did not contain mature stages in the kidney tubules at the time point of investigation.

As *T. bryosalmonae* can infect a variety of fish species, it can be assumed that an unspecific mode of host invasion is present for this parasite. To date, only a few details are known about the attachment and penetration of malacosporean spores upon contact to the fish host. Therefore, it were aims of the present study to clarify, which morphological features distinguish the spores released from *T. bryosalmonae*–infected bryozoans from other myxozoan species, to determine how the attachment and penetration to the fish host takes place and where the portal of entry into the fish is located. To characterize these early stages of the life-cycle ultrastructurally, infection trials and *in vitro* experiments with bryozoa-spores of *T. bryosalmonae* were carried out. The required spores were obtained from the laboratory cycle of the parasite. To be able to collect a sufficient number of spores at one time, parts of bryozoan colonies containing spores or mature sac stages were dissected. For chemical activation of spores, trout mucus homogenate was used. To activate the spores mechanically, the spore suspension and the mucus homogenate were pipetted up and down. The reaction of the spores was observed microscopically on a microscope slide. To detect the portal of entry of *T. bryosalmonae* into the fish host SPF rainbow trout fry were exposed to 1,000 to 2,000 bryozoa-spores of the parasite in a small volume of water. This experiment was repeated several times with incubation times ranging from 1 min to 60 min. After incubation, fish were anesthetized and dissected. Gills and pieces of the skin were fixed for light microscopy and transmission electron microscopy. Additionally, gills, whole fish and spore suspensions were prepared for scanning electron microscopy (SEM). Thereby it was possible to investigate the morphology of bryozoa-spores by SEM for the first time. The enclosure of polar capsule cells

by valve cells, the small opening for the polar filament and the plug sealing the opening of the channel where the filament is released were observed. The amoeboid movement of the sporoplasms was visualized by the *in vitro* activation experiments. Immediately after activation, the sporoplasm cells left the spore valves and moved slowly by forming pseudopodia. These processes are known already for other myxozoans, but were not shown for malacosporeans so far. In light microscopy of immunohistochemically stained samples attached spores, penetrating sporoplasms and stages inside of the gill tissue were observed. The highest number of attached stages was found 20 and 30 min after exposure and the earliest time when stages were found inside of the gill was 20 min. In contrast to the gill, no parasite stages were found attached or inside of the epidermis of the skin. Therefore, the ultrastructural findings indicate that the gills are main portals of entry for this parasite. In light and electron microscopy it was shown that sporoplasms of *T. bryosalmonae* enter the body of the host directly through the gill epithelium and not through mucus cells like for example sporoplasms of *Myxobolus cerebralis*. In SEM no stages were found on the gills and the body surface of whole fish. This might be due to loss of attached stages during processing of the samples.

After penetration, the parasite most likely reaches the target organ via the blood stream. In the blood, stages are exposed to the immune system of the host. Thus, the number of penetrated stages and the host reaction against the parasite will influence its further development and multiplication. Genetic differences in strains of the vertebrate host might influence success of penetration and further development of the parasite. This variability in susceptibility was assessed in the present study, because reliable data on intra-specific differences of the reaction of the fish host against malacosporean parasites were missing. For this purpose, fingerlings of four rainbow trout strains and brown trout were infected with *T. bryosalmonae* under identical conditions. After 2, 3 and 4 wpe 8 fish of each strain/species were anesthetised, weighed and measured and dissected to take samples of the kidney for molecular biology and histo-chemical investigations to assess the intensity of infection. For the quantification of parasite stages a *T. bryosalmonae*-specific quantitative real-time PCR assay (q-RT-PCR) was developed. According to sequence information of *T. bryosalmonae* and other myxozoans in the GeneBank, specific primers were developed that amplify a 166 bp fragment of the 18S rDNA of the parasite. To allow quantification relative to the number of host cells, the already described primer pair specific for trout insulin-like growth factor 1 (IGF 1) gene was used. Reaction conditions were optimized for the primers and their efficiency was determined in

pre-trials. Q-RT-PCR was conducted with Sybr-Green detection. Analysis of the q-RT-PCR data showed that the brown trout used were more susceptible to the parasite than all rainbow trout strains tested. After the first sampling time points of the experiment 2 and 3 wpe, two rainbow trout strains showed a significantly lower parasite load than all other groups. Nevertheless, the parasite multiplied faster in these fish in the further course of the experiment. As a consequence, at the last sampling time point, 4 wpe, the relative infection rate was similar in all rainbow trout strains. By immunohistochemistry stages of *T. bryosalmonae* were detected in histological sections, but the number was too low to allow quantitative evaluation of parasite load. A weak negative correlation between size of the fish and parasite load was detected. In total, these results indicate that some rainbow trout strains were able to slow down the development of the parasite after the first weeks of infection. This might be due to a more efficient antibody mediated immune response. A further interesting result was that the comparatively *T. bryosalmonae*-resistant rainbow trout strain was previously found to be highly susceptible to *M. cerebralis,* another myxozoan parasite. Obviously, resistance against one myxozoan species does not allow conclusions about susceptibility to other representatives of the group.

Besides *T. bryosalmonae,* two more malacosporean species are described today, exclusively belonging to the genus *Buddenbrockia*. Knowledge about life-cycles of these species is incomplete as descriptions exist only from stages in the bryozoan hosts. In the present study the question was addressed, if Malacosporea of the genus *Buddenbrockia* have life-cycles involving a fish host like it is the case for *T. bryosalmonae*. For this purpose different bryozoan species were collected from a natural pond and checked for infection with sack- or worm-like malacosporean stages. By infected bryozoans of the species *Plumatella repens* cohabitations experiments with parasite free carp (*Cyprinus carpio*), minnow (*Phoxinus phoxinus*) and brown trout were conducted. Thereby it was possible to cause infection in both cyprinids, while no infection with *Buddenbrockia* spp. was found in brown trout. These totally unknown parasite stages were detected in the kidney of the fish. By comparison of a 1,600 bp fragment of the 18S rDNA, the parasite found in minnow was identified as *Buddenbrockia plumatellae* (99.5 % sequence similarity), while the carp parasite is most likely a new species of the genus *Buddenbrockia*. The highest similarity of an 18S rDNA sequence with the latter parasite isolate was found for *B. allmani* with 97.8 %. Single infected carp and minnow were dissected after different time points and samples of the kidney were taken. By light and electron microscopical investigations of the kidney samples, parasite

stages were detected in the kidney tubules of both fish species. In minnows, only single parasite cells were found, while in kidney tubules of carp numerous multicellular sporogonic stages were observed. The highest number of stages in carp kidney was detected 8 wpe. These developmental stages were surprisingly similar to fish stages described from *T. bryosalmonae*. Only three specimens of minnow were available for the experiment, therefore samples could be taken only until 5 wpe. So far, no presporogonic stages of the fish-infecting *Buddenbrockia* spp. were found, possibly indicating their location in another organ. Cohabitation of parasite-free, laboratory-raised bryozoans with *Buddenbrockia*-infected carp did not cause overt infection in the bryozoans. Only DNA of the parasite was detected in some parts of the colony. Therefore, the complete life-cycle of the parasite has still to be established to reliably prove the infection of bryozoans by infected fish. With the obtained information on sequences of 18S rDNA a phylogenetic analysis of the known *Buddenbrockia* spp. was conducted. A multiple alignment was created and a phylogenetic tree was calculated by Bayesian inference. The separation of the species *B. plumatellae* in two lineages, already suggested in previous studies, was supported by this analysis. The carp-infecting malacosporeans could not be reliably placed in the tree, because differences in the sequences were too low to yield a higher phylogenetic resolution.

The results of the present study substantially increased the knowledge about host spectrum, host-parasite interaction and developmental cycles of different, also presumably new malacosporean species. This was achieved by means of bryozoan cultures and the laboratory cycle of *T. bryosalmonae* that was established in this study. The finding that some susceptible salmonid species are able to transmit *T. bryosalmonae* to bryozoans, while others could not do so, corroborates the assumption that a European and a North American strain of *T. bryosalmonae* exist, differing in their spectrum of compatible hosts. Further studies are desirable to clarify this interesting aspect of the evolutionary biology of this parasite. Early penetration processes of *T. bryosalmonae* into the fish host were elucidated by the results obtained in the present study. For the first time, amoeboid movement of sporoplasms of *T. bryosalmonae* were shown *in vitro* and the penetration process of this parasite could be followed histologically. These results will help to assess the host and tissue specificity of malacosporean parasites in future studies. Furthermore, indications for differences in the resistance of different rainbow trout strains against *T. bryosalmonae* were found, though the mechanisms of these differences have still to be clarified. In connection to the experiments on host specificity of the parasite, it can be speculated that host-parasite co-evolution might lead to

differences in susceptibility and incomplete development of the parasite in non-adapted hosts. Most striking was the discovery of *Buddenbrockia* spp. that were able to infect fish hosts and to develop up to sporogony, similar to *T. bryosalmonae*. Nevertheless, the complete life-cycle of these parasites involving the infection of bryozoans has still to be shown. Additionally, it is of interest, if other species of the genus *Buddenbrockia* also require a fish host in their life-cycles.

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# 11. APPENDIX

## 11.1 Recipes for Buffers and Solutions

### 11.1.1 Algae WC-medium

Stock solutions

$CaCl_2$ $2{\cdot}H_2O$ ..... 8.40 g

$MgSO_4$ $7{\cdot}H_2O$ ..... 18.50 g

$NaHCO_3$ ..... 6.30 g

$K_2HPO_4$ $3{\cdot}H_2O$ ..... 5.70 g

$NaNO_3$ ..... 42.50 g

$Na_2SiO_3$ $5{\cdot}H_2O$ ..... 10.60 g

→ dissolve each separately in 500 mL distilled water

Vitamin mix

Thimamin HCl (B1) ..... 0.1 g

Biotin (H) ..... 0.5 mg

Cyanocobalamin (B12) ..... 0.5 mg

→ dissolve all together in 1L distilled water

Trace elements

Na2EDTA (Titriplex III) ..... 4.36 g

$FeCl_3$ $6{\cdot}H_2O$ ..... 3.15 g

$CuSO_4$ $5{\cdot}H_2O$ ..... 10 mg

$ZnSO_4$ $7{\cdot}H_2O$ ..... 22 mg

$CoCl_2$ $6{\cdot}H_2O$ ..... 10 mg

$MnCl_2$ $4{\cdot}H_2O$ ..... 180 mg

$Na_2MoO_4$ $2{\cdot}H_2O$ ..... 6 mg

$H_3BO_3$ ..... 1 g

→ dissolve all together in 1L distilled water

Final solution: Make 2 mL of each stock solution, the vitamin mix and the trace elements to 2 L with distilled water and autoclave.

### 11.1.2 Immunohistochemistry

DAB-solution

10 mg tablet of 3,3-diaminobenzidene tetrahydrochloride (DAB) dissolved in 6.7 mL TBS; 0.5 mL aliquots of this solution were stored at -20 °C.

Before use, solution was centrifuged to remove undissolved DAB crystals.

Final solution: 0.5 mL DAB + 5 mL TBS + 0.1 mL 1% $H_2O_2$.

P01 monoclonal antibody

200 µg antibody were reconstituted with 1 mL PBS, aliquoted to portions of 50 µL and stored at -80 °C. Before use, the antibody-stock was diluted 1:10 with TBS.

Phosphate buffered saline (PBS) (0.02 M phosphate; 0.15 M NaCl)

$NaH_2PO_4 \cdot 2\ H_2O$..... 0.876 g

$Na_2HPO_4 \cdot 2\ H_2O$..... 2.56 g

NaCl..... 8.77 g

Tris buffered saline (TBS)

Trisma base.....2.42 g/L

NaCl.....29.24 g/L

PH was adjusted to 7.2 with HCl.

### 11.1.3 Electron microscopy

Soerensen phosphoric buffer

Solution A: $KH_2PO_4$.....9.078 g/L

Solution B: $Na_2HPO_4$ .....11.867 g/L

for ph 7.4 mix 81.8 mL of solution B with 18.2 mL of solution A.

5% Uranyl acetate solution

Uranyl acetate ($UO_2(CH_3COO)_2$ $2{\cdot}H_2O$)..... 50 g/L

Cover with foil and stir overnight. Add 10 drops of glacial acetic acid.

Reynold's Lead Citrate Solution

Add chemicals in distilled water in the following order:

Lead nitrate ($Pb(NO_3)_2$)..... 26.6 g/L

Sodium citrate ($Na_3C_6H_5O_7$ $2{\cdot}H_2O$) ..... 35.2 g/L

(solution becomes cloudy when sodium citrate is added)

1N NaOH..... 100 ml

(solution becomes clear when NaOH is added)

Distilled water ..... 600 ml

Stir for 10 minutes to dissolve and add additional 300 mL of distilled water.

Toluidine blue

Distilled water ..... 1 part

5% Toluidine blue in dist. water (w/v)..... 1 part

2% Sodium borate ($Na_2(B_4O_5(OH)_4)$ $8{\cdot}H_2O$) in dist. water (w/v) . 1 part

Epon resin

A) Glycid ether 100 ..... 38.32 g

2-2-dodecyl succinyl anhydride ..... 45.30 g

B) Glycid ether 100 ..... 61.80 g

Methyl nadic anhydride..... 56.34 g

A:B = 7:13 + 1.5% 2,4,6-tridimethylamino mehtyl phenol (DMP) (v/v)

## 11.2 Staining Protocols

### 11.2.1 Haematoxylin and eosin stain

1. Dewaxing in xylene and hydration in a decreasing ethanol series to distilled water.
2. Staining in haematoxylin solution (Roth) for 3-5 min.
3. Running tap water for 10 min.

4. Wash in distilled water.
5. Counter stain with 1.5 % eosin in distilled water (w/v) for 3 min.
6. Wash in distilled water.
7. Dehydrate in an increasing ethanol series and clear in xylene.
8. Mount slides with cover slip.

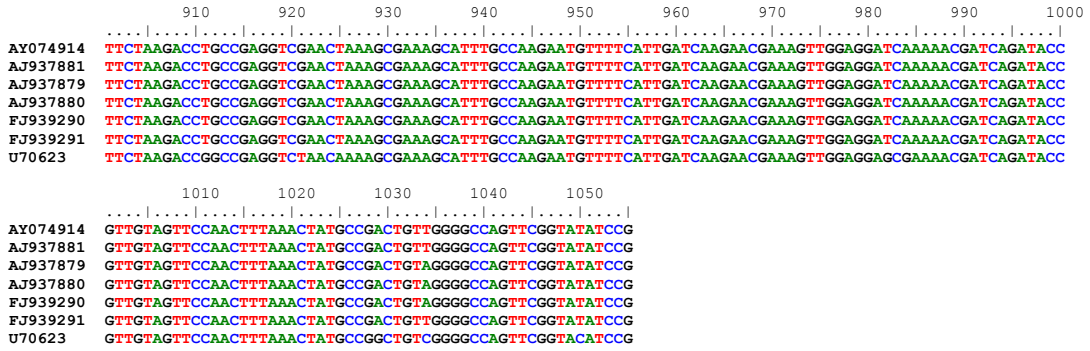
### 11.2.2 Diff-Quick

1. Air-dry blood smear.
2. Fix in Diff Quick Fixative (or methanol) for 30 secs/drain.
3. Stain with Diff Quick solution II for 30 secs/drain.
4. Counterstain with Diff Quick solution I for 30 secs/drain.
5. Rinse in tap water to remove excess stain.
6. Rapidly dehydrate in absolute alcohol.
7. Clear and mount.

### 11.2.3 EM contrasting

1. Grids are placed with the section down on a drop of uranyl acetate for 10 min.
2. Wash grids in distilled water.
3. Tap grids dry carefully with filter paper.
4. Place grids on drop of lead citrate for 1 min.
5. Tap grids dry carefully with filter paper.
6. Store grid in excicator.

## 11.3 Multiple Alignment of *Buddenbrockia* 18S rDNAs




AY074914: *Buddenbrockia plumatellae* (worm-shape)

AJ937881: *B. plumatellae* (sac-shape)

AJ937879: *Buddenbrockia sp.* (from *Fredericella sultana*)

AJ937880: *B. allmani*

FJ939290: *Buddenbrockia sp.* (from carp)

FJ939291: *Buddenbrockia sp.* (from minnow)

U70623: *Tetracapsuloides bryosalmonae*

# 12. LEBENSLAUF


**Persönliche Daten**

| | |
|---|---|
| Name | Daniel Grabner |
| Geburtsdatum | 06. Juli 1978 |
| Geburtsort | Dresden |
| Nationalität | Deutsch |
| Familienstand | Ledig |

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**Werdegang**

| | |
|---|---|
| 11.2006 – 11.2009 | Promotionsarbeit an der Klinik für Fische und Reptilien (ehemals Institut für Zoologie und Fischereibiologie) und der Klinik für Geflügel, Ziervögel, Reptilien und Fische der Veterinärmedizinischen Universität Wien: „Investigations on life-cycle and host specificity of the Malacosporea (Myxozoa)“, (Leitung: Prof. El-Matbouli in Kooperation mit Prof. Sures, Universität Duisburg/Essen). |
| 12.2004 – 09.2005 | Diplomarbeit in der Physiologischen Ökologie der Tiere an der Eberhard-Karls Universität Tübingen: „Suborganismische Effekte von Nickelchlorid auf frühe Lebensstadien des Zebrabärblings *Danio rerio* – Histopathologie und Stressproteine“ (Leitung: Prof. H.-R. Köhler). |
| 10.1999 – 07.2005 | Studium der Biologie an der Eberhard-Karls Universität Tübingen<br>Hauptfach: Zoologie<br>Nebenfächer: Geoökologie und Ethik in den Biowissenschaften<br>Akademischer Grad: Diplombiologe<br>Abschlussnote: 1.1 |
| 09.1998 – 10.1999 | Zivildienst bei der Körperbehindertenförderung (KBF) in Reutlingen.<br>Tätigkeit in betreuter Wohnanlage. |

**Schulabschluss**

| | |
|---|---|
| 06.1998 | Allgemeine Hochschulreife am Albert-Einstein-Gymnasium Reutlingen<br>Abschlussnote: 2.1 |

**Arbeitsverhältnisse**

| | |
|---|---|
| Seit 01.07.2009 | Wissenschaftlicher Mitarbeiter an der Klinik für Geflügel, Ziervögel, Reptilien und Fische der Veterinärmedizinischen Universität Wien.<br>Weiterführung des Projektes Lebenszyklus, Wirtsspezifität und -Invasion von *Tetracapsuloides bryosalmonae* (Erreger der PKD bei Salmoniden) bis voraussichtlich 30.11.09 (Leitung: Prof. El-Matbouli). |
| 05.2009 – 06.2009 | Beschäftigung im Rahmen eines Forschungsstipendiums an der Klinik für Fische und Reptilien, LMU München zur Weiterführung der Promotionsarbeit. |
| 10.2007 – 04.2009 | Wissenschaftlicher Mitarbeiter an der Klinik für Fische und Reptilien (ehemals Institut für Zoologie und Fischereibiologie), LMU München im Rahmen des DFG-geförderten Forschungsprojekts zu Lebenszyklus, Wirtsspezifität und -Invasion von *Tetracapsuloides bryosalmonae* (Leitung: Prof. El-Matbouli). |
| 06.2007 – 09.2007 | Wissenschaftliche Hilfskraft im Institut für Zoologie und Fischereibiologie, LMU München zur Betreuung der Fischbestände. |
| 11.2005 – 11.2006 | Wissenschaftlicher Mitarbeiter am Institut für Zoologie und Fischereibiologie der Ludwig-Maximilians Universität (LMU) München im Rahmen eines DFG-geförderten Forschungsprojektes.<br>Schwerpunkt: Lektin-Glykoprotein-Wechselwirkungen bei parasitären Invasionsvorgängen (Leitung: Prof. El-Matbouli). |

**Weitere Tätigkeiten im Rahmen der Arbeitsverhältnisse**

- Lehrtätigkeit im Rahmen der Zoologievorlesung für Tiermediziner und Durchführung von Zoologiekursen (Protozoen, Insekten, Fische).
- Mitarbeit bei der Fischdiagnostik.
- Verwaltung des Vorlesungsverzeichnisses und der Teilnahmelisten für Wahlpflichtfächer.
- Verwaltung der Institut-Homepage.

**Zusatzqualifikationen**

- Sprachkenntnisse
  - Englisch fließend in Wort und Schrift.
  - Französisch Grundkenntnisse.
- EDV-Kenntnisse
  - Routinierter Umgang mit MS Word, Excel und Powerpoint.
  - Bild- und Grafikverarbeitung, sowie -erzeugung mittels Photoshop, GIMP und Corel Draw.

- Sicherer Umgang mit Statistiksoftware: SPSS und GraphPad.
- Routinemäßige Verwendung diverser Bioinformatiksoftware für molekularbiologische Arbeiten.

- Drittmittelerwerb: Erfahrung mit Forschungsmittelbeantragung (DFG).

**Ehrenamtliche Tätigkeiten**

- Abteilungsleiter der Badmintonabteilung der TSG-Reutlingen (2001 – 2006).
- Zweitweise Jugendtrainer und Pressewart bei der Badmintonabteilung der TSG-Reutlingen.

Wien, 01.11.2009

.....

Daniel Grabner

# 13. ERKLÄRUNGEN

**Erklärung:**

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung der Dr. rer. nat., dass ich das Arbeitgebiet, dem das Thema „**Investigations on life-cycle and host specificity of the Malacosporea (Myxozoa)**“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Daniel Grabner befürworte.

Essen, den _____

Prof. Dr. Bernd Sures

**Erklärung:**

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Wien, den _____

Daniel Grabner

**Erklärung:**

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Wien, den _____

Daniel Grabner